Thesis for doctoral degree (Ph.D.)
2019

# Human Amnion Epithelial Stem Cells as a Therapy for Liver Disease

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Stockholm 2019

Published by Karolinska Institutet.
Printed by Arkitektkopia AB

ISBN 978-91-7831-533-8

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

The thesis will be defended at Månen 9Q, Alfred Nobels allé 8 (Floor 9), Flemingsberg Campus

Friday, October 18, 2019, at 9:00

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**வாழ்க தமிழ்** **வளர்க தமிழ்**

Thirukural/திருக்குறள் - அதிகாரம்/Chapter: கல்வி / Learning

**கேடில் விழுச்செல்வம் கல்வி யொருவற்கு**
**மாடல்ல மற்றை யவை.**

Learning is the true imperishable riches; all other things are not riches.

# ABSTRACT

Placenta-derived stem cells have been proposed as potential new treatments for acute and congenital liver diseases. Of all the different perinatal tissues, amnion membrane and isolated amnion epithelial cells have been shown to be an outstanding readily available source of multipotent stem cells. Human amnion epithelial cells (hAEC) have unique properties, including low immunogenicity and immunomodulatory properties, which may allow the first allogenic stem cell therapy without immunosuppression. Animal studies have shown that hAEC differentiate into hepatocyte-like cells and support missing liver functions commonly responsible for inborn errors of metabolism. In the present thesis, we describe early preclinical steps which will likely be necessary to translate hAEC therapy into clinical practice. These steps include detailed and optimized methods for primary hAEC isolation and preservation, methods to validate the final cell product and investigations of the route of infusion for efficient engraftment in the target organ (liver). The efficacy of hAEC transplants was assessed in preclinical models of liver disease.

In Project 1, we have detailed the hAEC isolation procedure with GMP reagents, providing a homogenous amnion epithelial cell suspension. The preclinical validation of hAEC-based therapy was continued in Project 2, where 14 different batches of primary hAEC were characterized by immunocytological and biomolecular techniques. The presented findings indicate this technology results in an enriched suspension of epithelial cells with a minimal contamination with mesenchymal, endothelial or hematopoietic cells. In Project 5, we validated the route of infusion of hAEC to reach high level of engraftment in liver. We investigated the bio-distribution of injected DiR-labelled hAEC administered via tail-vein or intra-splenic, and monitored their localization using *in vivo* live imaging (IVIS) techniques. Twenty-four hours post-splenic infusion, the majority of hAEC was safely delivered and detected in the liver parenchyma. On the contrary, tail-vein infusion resulted in a wide distribution pattern to multiple organs.

In Project 3, we have investigated the *in vivo* engraftment, long-term survival and hepatic maturation of hAEC. We have injected hAEC into a metabolic liver disease model of Phenylketonuria (PKU). This immune-competent *PAH*-deficient mouse develops a pathological level of phenylalanine (PHE) in the blood, which is commonly observed in PKU patients. We assessed hAEC engrafted into murine liver parenchyma out to 100 days. Such long-term survival resulted in significant correction of blood PHE levels in blood and a statistical complete correction or PHE levels in the brain. The described xeno-transplantation was carried out without any immunosuppressant regimen, and no signs of rejection were noticed.

Problems generating clinically relevant results by extrapolation of data from mouse models was also addressed in Project 4, we successfully generated a liver-humanized mouse model that faithfully reproduces the metabolic liver disease observed in patients. We injected hepatocytes isolated from a CPS1 deficient patient into immune-compromised mice (FRGN), where primary human hepatocytes have been previously reported to engraft and fully repopulate the mouse liver. The resultant chimeric CPS1-Deficient (CPS1-D) model exhibited high blood ammonia levels, elevated disease-correlated amino acids (glutamine and glutamate) and low CPS1 enzymatic activity.

In conclusion, during the past 4-year study we have successfully analyzed pre-clinical data and validated the hypothesis that human amnion epithelial cells are useful for the cellular therapy of liver disease, supporting their potential to become a therapeutic tool to treat and support metabolic liver disease patients.

# LIST OF SCIENTIFIC PAPERS

1. Isolation of Human Amnion Epithelial Cells According to Current Good Manufacturing Procedures. Roberto Gramignoli, **Raghuraman C. Srinivasan**, Kristina Kannisto, Stephen C. Strom. Current Protocols in Stem Cell Biology, 2016, 37:1E.10.1-1E.10.13. doi: 10.1002/cpsc.2

2. Effect of Cryopreservation on Human Amnion Epithelial Cells. **Raghuraman C. Srinivasan**, Roberto Gramignoli, Kristina Kannisto, Stephen C Strom. Unpublished manuscript.

3. Amnion Epithelial Stem Cell Correction Of A Phenylalanine Hydroxylase Deficient Mouse Model. Kristen J. Skvorak, Roberto Gramignoli, Kenneth Dorko, Steven F. Dobrowolski, Kayla Spridik, Kristina Kannisto, **Raghuraman C. Srinivasan**, Toshio Miki, Jerry Vockley, Stephen C. Strom. Manuscript under revision.

4. A Liver Humanized Mouse Model of Carbamoyl Phosphate Synthetase 1 – Deficiency**. Raghuraman C. Srinivasan**, Mihaela Zabulica, Christina Hammarstedt, Tingting Wu, Roberto Gramignoli, Kristina Kannisto, Ewa Ellis, Ahmad Karadagi, Ralph Fingerhut, Gabriella Allegri, Véronique Rüfenacht, Beat Thöny, Johannes Häberle, Jean-Marc Nuoffer, Stephen C. Strom. Journal of Inherited Metabolic Disease, 2019, doi: 10.1002/jimd.12067

5. Evaluation of different routes of administration and biodistribution of human amnion epithelial cells in mice. **Raghuraman C. Srinivasan**, Kristina Kannisto, Stephen C. Strom, Roberto Gramignoli. Cytotherapy, 2019. doi: https://doi.org/10.1016/j.jcyt.2018.10.007

# LIST OF ADDITIONAL PAPERS

1. Guide to the assessment of mature liver gene expression in stem cell-derived hepatocytes. Mihaela Zabulica, **Raghuraman C. Srinivasan**, Massoud Vosough, Christina Hammarstedt, Tingting Wu, Roberto Gramignoli, Ewa Ellis, Kristina Kannisto, Alexandra Collin de l'Hortet, Kazuki Takeishi, Alejandro Soto-Gutierrez, Stephen C. Strom. Stem Cell and Development, 2019. https://doi.org/10.1089/scd.2019.0064.
2. Ectonucleotidase Expression on Human Amnion Epithelial Cells: Adenosinergic Pathways and Dichotomic Effects on Immune Effector Cell Populations. Fabio Morandi, Alberto L. Horenstein, Valeria Quarona, Angelo Corso Faini, Barbara Castella, **Raghuraman C. Srinivasan**, Stephen C. Strom, Fabio Malavasi and Roberto Gramignoli. The Journal of Immunology, 2018. doi:10.4049/jimmunol.1800432

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADO | Adenosine |
| ALF | Acute Liver Failure |
| AM | Amniotic Membrane |
| APC | Allophycocyanin |
| ATMP | Advanced Therapy Medicinal Product |
| BCAA | Branched Chain Amino Acids |
| BCKA | Branched-Chain-Aketo Acid |
| BCKDH | Branched-Chain-Aketo Acid Dehydrogenase |
| BPD | Bronchopulmonary Dysplasia |
| CBMPS | Cell-Based Human Medicinal Product |
| CPS1-D | Carbamoyl Phosphate Synthetase 1-Deficiency |
| D - Gal | D-Galactosamine |
| DC | Dendritic Cells |
| Dir | (1,1'-Dioctadecyltetramethyl Indotricarbocyanine Iodide |
| DMSO | Dimethyl Sulfoxide |
| EGTA | Ethylene Glycol Tetraacetic Acid |
| EpCAM | Epithelial Cell-Adhesive Molecule |
| ESC | Embryonic Stem Cells |
| Fasl | Fas Ligand |
| FIAU | Fialuridine |
| FITC | Fluorescein Isothiocyanate |
| GMP | Good Manufacturing practices |
| hAEC | Human Amnion Epithelial Cells |
| HE | Hematoxylin And Eosin |
| HIV | Human Immunodeficiency Virus |
| HLA | Human Leukocyte Antigens |
| HTx | Hepatocyte Transplantation |
| IDO | Indoleamine 2,3-Dioxygenase |

| | |
|---|---|
| IFN-γ | Interferon Gamma |
| IGL | Institut Georges Lopez |
| IPs | Induced Pluripotent Stem Cells |
| MIF | Macrophage-Inhibitory Factor |
| MRI | Magnetic Resonance Imaging |
| MSUD | Maple Syrup Urine Disease |
| Nir | Near Infra-Red Dye |
| NK | Natural Killer |
| NOD | Non-Obese Diabetic |
| NTBC | (2-(2-Nitro-4-Trifluoro-Methylbenzoyl)1,3-Cyclohexedione) |
| OCT-4 | Octamer Binding Protein 4 |
| OLT | Orthotopic Liver Transplantation |
| PAH | Phenylalanine Hydroxylase |
| PE | Phycoerythrin |
| PGE2 | Prostaglandin E2 |
| PKU | Phenylketonuria |
| SOX-2 | Sex Determining Region BOX 2 |
| SSEA | Stage Specific Embryonic Antigens |
| TERT | Telomerase Reverse Transcriptase |
| TGF-ß | Transforming Growth Factor-Beta |
| TIMPS | Tissue Inhibitors Of Metalloproteinases |
| TRA | Tumor Rejection Antigens |
| UW | University Of Wisconsin |

# 1 INTRODUCTION

## 1.1 Liver

The liver is a vital organ. One cannot survive without a liver for more than a few hours. The liver is vital, because it performs many functions critical to sustain life including the regulation of blood glucose, ammonia metabolism and urea production, the synthesis and secretion of plasma proteins including albumin and clotting factors and the metabolism and excretion of many endogenous substrates such as hormones, as well as xenobiotics (1) (Figure 1). Regenerative medicine is a term widely used to describe the biomedical approach to heal damaged tissue by either replacement of damaged cells, as with cell transplant techniques, or stimulating the repair activity of endogenous cells. Because of its extensive capacity to regenerate, the liver perhaps, more than any other organs offers a great opportunities in regenerative medicine. The primary cell type that performs the majority of liver functions is the parenchymal hepatocyte, which comprises approximately 60% of the cell number, and account for about 80% of the liver mass (1). The remaining, non-parenchymal cells include cholangiocytes (bile duct cells), Kupffer, stellate and endothelial cells. The liver also produces around 700ml of bile (2). This bile is collected in the bile duct and carried to the duodenum of the small intestine directly or via the gall bladder. Bile helps to breakdown and absorb fat in our diet.

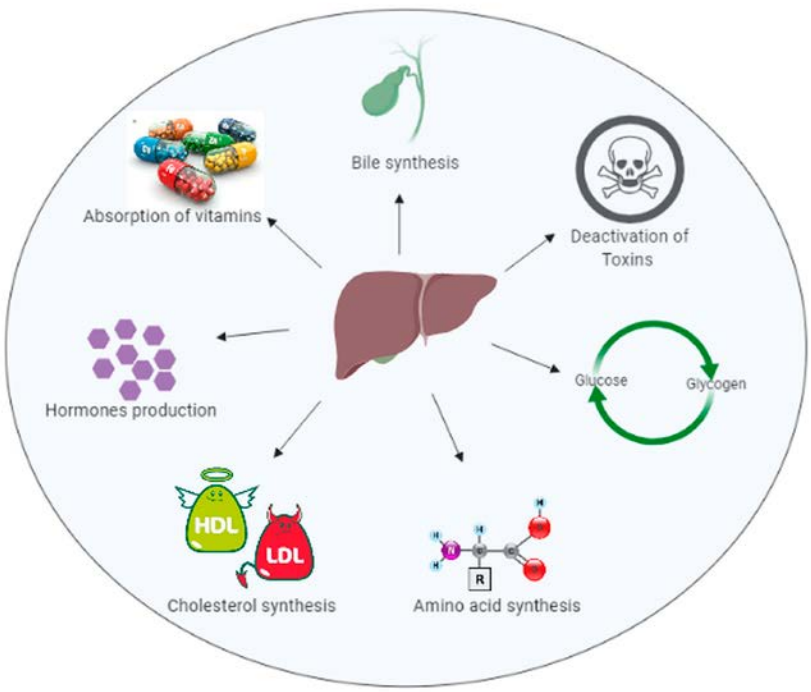


***Figure 1.*** *Pictorial representation of liver functions. Created with Biorender.com*

# 2 LIVER DISEASE

## 2.1 Liver Cirrhosis

Liver cirrhosis is characterized by deformity of the liver architecture, necrosis of hepatocytes and regenerative nodule formation. Cirrhosis is a late stage of scarring of liver that is common to many liver diseases (3). Major reasons include excessive alcohol consumption, viral hepatitis B or C, although there are other causes. Cirrhosis involves loss of liver cells and results in irreversible scarring of the liver. Orthotopic liver transplantation (OLT) is the ultimate solution for end stage liver disease. However, hepatocyte transplantation (HTx) pioneered by our laboratory is used as a bridge therapy for patients, who are awaiting for organ transplant. The liver architect is altered in liver cirrhotic patients, which makes HTx more complicated.

## 2.2 Acute liver failure (ALF)

Acute liver failure (ALF) remains a condition with considerable mortality (4) and is a catastrophic illness that may lead to severe hepatic injury or massive necrosis, hepatic encephalopathy, and frequently, death (5). The term fulminant hepatic failure applies to patients who develops hepatic encephalopathy within 2 months of the onset of liver disease such as jaundice (6). The chances of survival is mere 20%, where the only cure is OLT. Both viral hepatitis and drug induced liver injury are common reasons for ALF.

## 2.3 Metabolic liver disease

Since many liver functions are necessary to sustain life, mutations in the genes that encode these liver proteins / enzymes can be life-threatening. Metabolic liver diseases can result when these critical liver functions are deficient or completely absent. Most metabolic liver diseases are the result of a single protein or enzymatic defect, with the remaining hepatic activities being carried out at normal levels (7). For example, a patient with phenylketonuria (PKU) has a mutation in the *PAH* gene which prevents the conversion of phenylalanine into tyrosine. All the remaining functions such as clotting factors or drug metabolism are performed at normal levels. Historically, severe metabolic liver diseases have been treated by HTx (8, 9). The replacement of an entire liver to correct a single metabolic defect might seem excessive if there were other options to correct the defect which were equally effective but yet, less invasive. Here is where the concept of cellular therapy of liver disease began to be considered.

# 3 ORTHOTOPIC LIVER TRANSPLANTATION (OLT)

OLT has been the primary treatment for several end stage liver diseases and life threatening metabolic liver diseases for several decades. Despite the unquestioned success of OLT, the technique requires major surgery and takes a considerable recovery period (10).

The shortage of organ donors and other surgical complications, immune rejection and infections post-transplantation are all limitations for OLT (11). Cell therapy has been proposed as an alternative treatment for liver diseases (12, 13). Cell transplantation is far simpler than OLT, it is less expensive, less invasive and the patient keeps their native liver. Thus, in a situation where the cell graft is lost through senescence or rejection, the patient is simply returned to the pre-transplant state (Figure 2). Perhaps the prime benefit of cellular therapy is that since the patient keeps their native liver, the donor cells need only support the one liver function missing in the recipient, and are not required to support the entire range of liver functions. Some of the benefits of cellular therapy are listed in Figure 2.

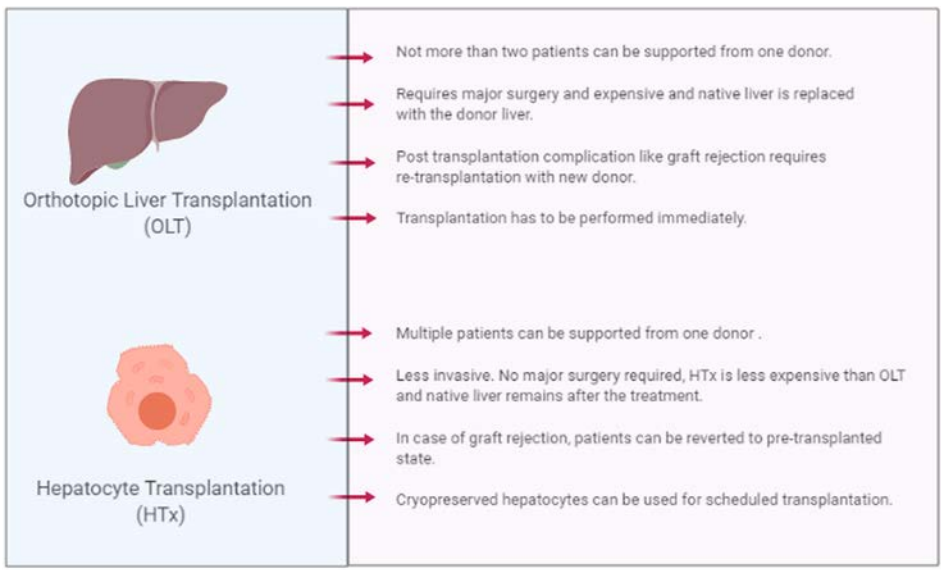


***Figure 2.*** *Advantages of cellular therapy of liver disease. Created with Biorender.com*

# 4 CELL-BASED THERAPY

## 4.1 Hepatocyte transplantation (HTx)

The liver parenchymal cells are the preferred choice of cells for the cell therapy of liver disease. More than 20 years of preclinical studies with small animal models have shown the safety and efficacy of hepatocyte transplants in the treatment of a variety of liver diseases. The first HTx in patients were carried out during the early 1990s (9, 12). Transplanted hepatocytes support the regeneration of the native liver and can also provide proteins or enzymatic activities missing in the recipient. The waiting time for patients to receive a suitable organ may vary from months to years and the numbers of patients that could benefit from organ replacement far exceed the availability of the donor organs. Thus, additional therapies are needed. HTx can bridge the patient and provide temporary liver support while waiting for a solid organ transplant. However, even with HTx, there are not enough useful livers available for cell isolation. This limitation with hepatocytes has led to a search for alternative sources of cells for this therapy.

## 4.2 Other cell sources

Multiple cell sources were proposed as an alternative source of cells for hepatocyte transplants. Among them, fetal hepatocytes, domino transplants, embryonic stem cells (ESC), induced pluripotent stem (iPS) cells, and human amnion epithelial cells (hAEC). Since even a few remaining undifferentiated pluripotent stem cells are capable of forming a tumor upon transplantation, the use of ESC or iPS cells remain at the preclinical stage with respect to liver disease. Also, there are no protocols published that describe the generation of hepatocyte-like cells from these stem cells with fully mature liver functions (14). Fetal hepatocytes do not express mature liver functions and are associated with some ethical concerns for their collection and use. Domino hepatocyte transplants have been conducted on two patients with PKU with cells isolated from patients with different metabolic liver diseases, but this source of cells, while useful, will not provide the numbers of cells needed for cellular therapy of liver disease (13, 15).

# 5 PLACENTA

The close relationship between the fetus and the mother is an important phenomenon in human development. The fetus has to establish an intimate relationship with the mother in order to get enough nutrients, oxygen and to discard waste products. The placenta is the medium that helps the fetus and importantly, protects the fetus from the mother's immune system; otherwise, it would be rejected since the fetus is a semi-allograft (16).

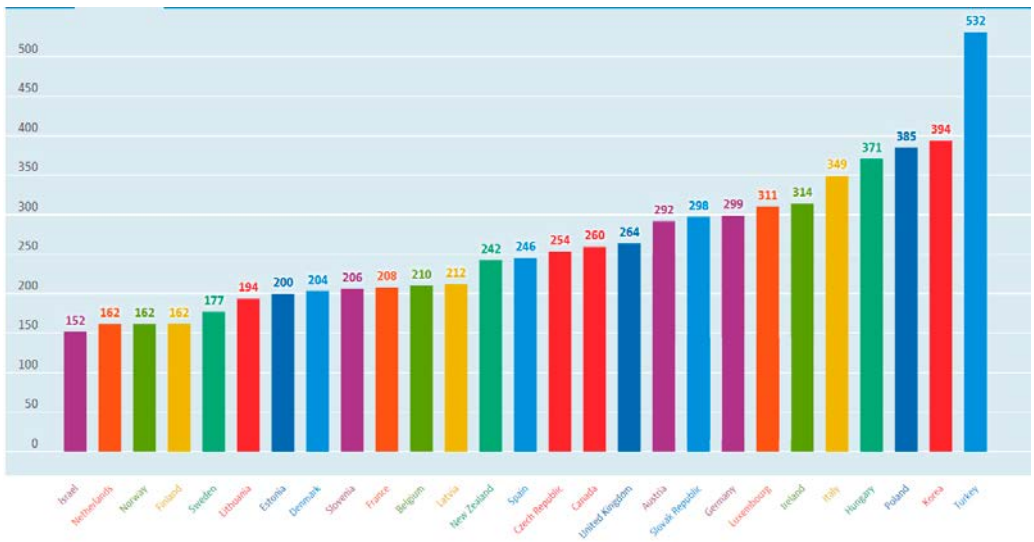


***Figure 3.*** *The numbers of caesarian-section procedures occurring in different countries per 1000 births. Data were obtained from the Organization for Economic Co-operation and Development (OECD) countries and are according to 2016 statistics.*

The placenta is a tissue that provides nutrients and oxygen exchange between the fetus and mother through the umbilical cord (16). The proper function of the placenta is crucial for a successful, full-term pregnancy. The placenta is dark reddish in color with 20-22 cm in length, 2-2.5 cm in thickness and usually weighs around 500-600g. The placenta is composed of three layers: amnion, chorion, and decidua respectively (Figure 4).

Amnion and chorion are membranes of fetal origin whereas decidua is a maternal tissue adherent to the chorion membrane. Amnion membrane continues from the edge of the placenta and encloses the amniotic fluid and the fetus (16). Several types of stem cells can be isolated from full-term placentae such as human amnion epithelial cells, human amnion mesenchymal stromal cells, chorionic mesenchymal stromal cells, and hematopoietic stem cells (17).

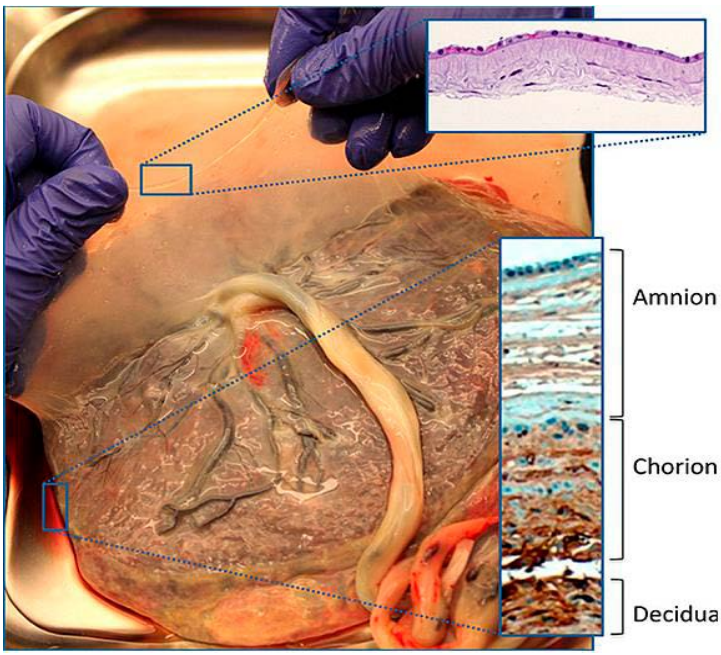


***Figure 4.*** *Human placenta. The amnion membrane getting separated from the chorion layer. The amnion membrane stained with H&E (top panel) and histology section of three layers of placenta (bottom panel). (R. Gramignoli, Curr Pathobiol Rep 2016. 4:157-167)*

Advantages of using placental derived stem cells is the lack of ethical concerns. Unlike embryonic stem cells, the ethical problems can be overcome with these placenta derived cells since the placenta is usually discarded after delivery.

## 5.1 Amnion membrane

By approximately day 8 after fertilization, the inner cell mass begins to segregate into epiblast and hypoblast layers. Concomitant with this, the amnion epithelial cells differentiate from the epiblast (Figure 5) (18). The amnion cavity develops within the epiblast and the cells adjacent to the amniotic cavity, termed amnioblasts, proliferate and finally become a thin, tough, elastic, avascular amniotic membrane (AM ) (19). AM has several favorable properties for clinical applications such as anti-fibrotic, anti-inflammatory and pro-regenerative effect (20). The AM can be used before and after cryopreservation without any difference in their clinical potential features (20). AM has been used for patching liver to counteract liver fibrosis (20), wound healing such as fire burns (21), also used to promote corneal healing in severely damaged eye (22).

The AM is an uninterrupted single layer of columnar epithelial cells in contact with amniotic fluid. The AM can be subdivided into five anatomic layers: the epithelial cell monolayer, an acellular basement membrane layer, a compact layer (basal lamina), dispersed stromal cells (mesenchymal cell layer) and a spongy layer which is located close to chorionic membrane.

Both the amnion epithelial and mesenchymal cells possess stem cell characteristics and immunomodulatory properties which has attracted the attention for regenerative medicine applications (18).

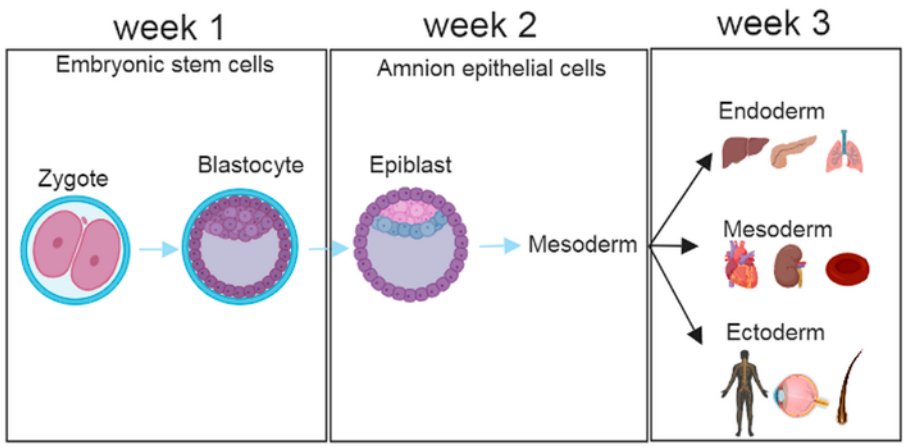


***Figure 5.*** *Embryogenesis from fertilization to gastrulation. Created with Biorender.com*

# 6 HUMAN AMNION EPITHELIAL CELLS

## 6.1 Origin

The hAEC have several features that make them attractive for cell therapy. The availability of hAEC is plentiful, and their collection and use are non-controversial (18, 23). In the field of regenerative medicine, there are many potential sources of cells available for clinical studies and application, such as ESC or iPS. Although their biological potential have been demonstrated, still these cells are not widely accepted for clinical treatments.

The origin of hAEC makes them unique and these cells possess plasticity that allows them to differentiate into all three germ layers (18). In special conditions, *in vitro*, the hAEC can differentiate into ectoderm (neural cells) (24); mesoderm (cardiomyocyte) (18, 23); endoderm (liver and pancreas) (23). There are numerous reports of hAEC adopting hepatic characteristics, *in vitro,* or upon transplantation, *in vivo*. Ours, and several other groups have reported the expression of genes that are normally expressed in the mature liver (25, 26). Properties listed in Figure 6 support the use of hAEC for regenerative medicine.

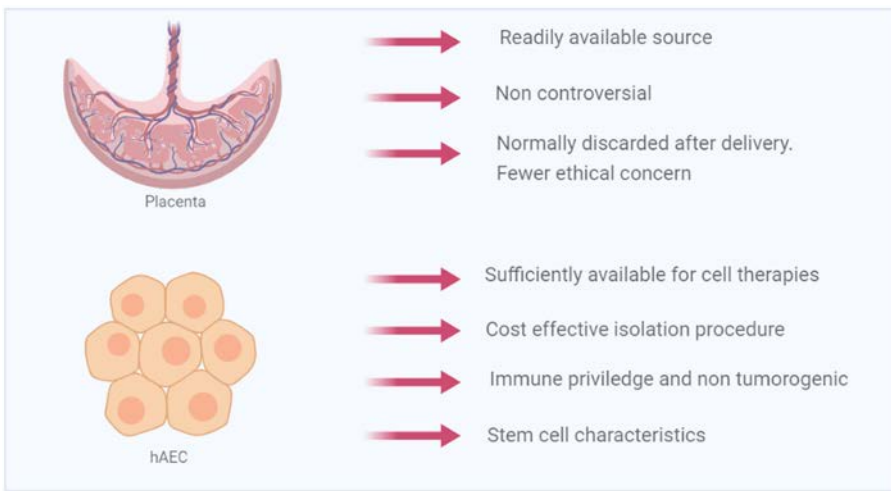


***Figure 6.*** *The advantages of using human amnion epithelial cells in regenerative medicine. Created with Biorender.com*

## 6.2 Stem Cell Properties

The origin of amnioblast from the epiblast happens prior to gastrulation, the point where the three germ layers are specified (27). This unique development of hAEC prior to tissue specification may explain their multipotent nature.

The hAEC express surface markers that are also normally expressed in embryonic stem cells including tumor rejection antigens (TRA) 1-60 and 1-81. The hAEC also express stage-specific embryonic antigens (SSEA)-3 and -4 (18). Apart from these surface markers, hAEC were also reported to express genes commonly expressed in pluripotent stem cells, including Octamer binding protein 4 (OCT-4), sex-determining region BOX 2 (SOX-2) and NANOG; genes are known to be important for maintaining pluripotency (18).

## 6.3 Tumorigenicity

Tumorigenicity is one of the major hindrances in using pluripotent stem cells in cell-based regenerative medicine therapies, since they may cause formation of tumors due to their immortal nature. On the other hand, hAEC do not express telomerase reverse transcriptase (TERT), they are not immortal and do not form tumors upon transplantation (unlike ESC and iPS). Tumorigenicity of hAEC was evaluated by injecting 1 million hAEC in large numbers of SCID mice that were kept under observation for 7 months (18).

## 6.4 Immune Privilege

Immune rejection is a major limitation for liver transplantation. Under normal conditions, the host's immune system will identify the transplanted cells as foreign and will initiate an immune response to eliminate them. The Immune privilege status of hAEC may break barriers in cell therapy and may save patients from lifelong immunosuppressive drugs (28). Human Leukocyte antigens (HLA) plays a major role in the immune system. They are divided into two types, HLA I and HLA II respectively. The HLA molecules are referred to as 'transplantation antigens', and their presence on the transplanted cells may lead to immune-mediated rejection (29).

### 6.4.1 Low Immunogenicity

When transplanted, hAEC have displayed low immunogenicity. Nature has evolved a mechanism to protect the fetus from the mothers' immune system (Figure 7). Generally, rejection of cells or organs occurs due to mismatch of HLA expression from the host immune system. Unique characteristics of hAEC are that they express low levels of HLA class Ia molecules; HLA A, B, C, and express significant levels of less common HLA class Ib molecules -E, -F, -G which are potent immunomodulators. They do not express human leukocyte antigen class II (HLA-DP, DQ and -DR) antigens and the co-stimulatory molecules, CD40 and CD80 (16, 30).

To examine their immunogenicity and survival, hAEC were transplanted into seven volunteers, six men and one woman, by Akle *et al* 1981. There was no sign of acute rejection and the survival of hAEC was demonstrated out to 7 weeks post-transplantation by biopsy (31).

### 6.4.2 Human Leukocyte Antigen-G

The fetus is semi-allograft since it contains the genetic material from the father and the mother. In normal conditions, even semi-allografts would be considered as foreign and rejected by the host immune system. However, under normal conditions the fetus can grow within the mother for the entire duration of pregnancy without immune-related problems or rejection. One of the major reasons for this tolerance is thought to be HLA-G, a key factor for fetal-maternal tolerance. HLA-G is a non-classical, non-polymorphic HLA class 1b antigen that is critical in regulating the tolerance of the fetus by the maternal immune system (32).

HLA-G has been identified as a ligand for two inhibitory receptors namely, ILT2 and ILT4 (CD85j and CD85d) and the receptor KIR2DL4/CD158d (33-35). The receptor KIR2DL4/CD158d is a HLA-G specific receptor and ILT2 and 4 have the highest affinity for HLA-G (36). It is commonly believed that these three receptors for HLA-G play an inhibitory role on the immune system. Natural killer (NK) cells express the KIR2DL4 receptor and it can act as an activating receptor on NK cells and results in upregulation of IFN-γ production (36). Also, interferon-gamma (IFN-γ) can provoke the the immunosuppressive effect of hAEC by inducing indoleamine 2, 3-dioxygenase (IDO) activity.

Because of these direct and indirect effects in immune regulation, HLA-G has been proposed as a therapy for solid organ and cell transplantation. HLA-G is present in and on hAEC both in a soluble, as well as membrane-bound form.

### 6.4.3 Immunomodulatory Effect

In addition, hAEC also directly or indirectly modulates the inflammatory signals that result in tissue rejection. Amnion epithelial cells derived factors have the ability to inhibit neutrophils and macrophages *in vitro* (37). Macrophage-inhibitory factor (MIF), which is a potent inhibitor of macrophage migration is released by hAEC. It was also shown that MIF is a potent inhibitor for natural-killer cells mediated lytic activity (38). When T and B cells were incubated with hAEC supernatant, there is 6-7 fold increase in T and B cell apoptosis when compared with untreated controls (37). Dendritic cells (DC) plays an important role in presenting antigens to T cells. Monocytes exposed to DC maturation in the presence of amniotic membrane-derived cells results in impaired development of DC (39).

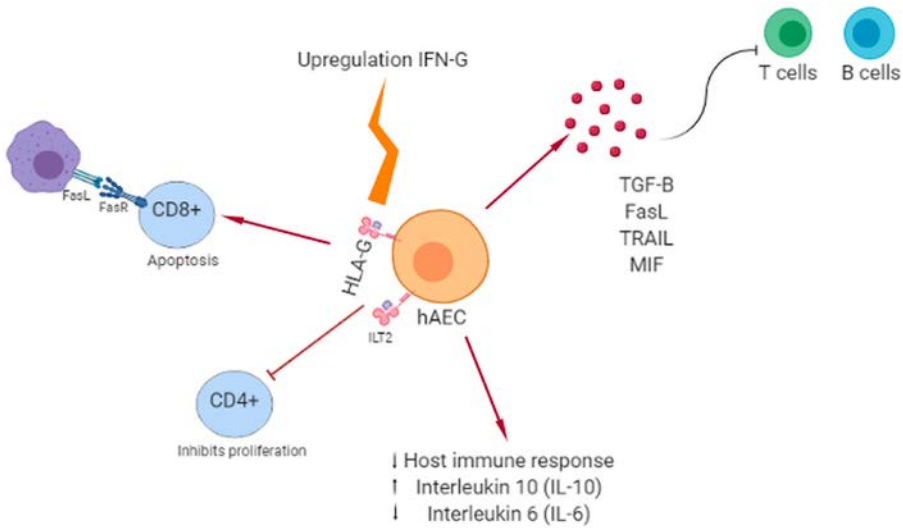


***Figure 7.*** *Immunomodulatory/Immunosuppressive properties of hAEC. The hAEC express several factors to suppress T and B cells proliferation such as TGF- ß, FasL, TRAIL, MIF. It also expresses HLA-G, which suppresses the proliferation of CD4+ cells and induce apoptosis in CD8+ cells. Also, FasL binds to the FasR on activated the host immune cells which results in cell apoptosis. The hAEC are known to modulate the host immune system by increasing IL-10, IL-6 and other cytokines. Created with Biorender.com*

The hAEC secrete other immunosuppressive factors such as prostaglandin E2 (PGE2), which has several immunosuppressive properties such as inhibition of T cell proliferation. PGE2 stimulates the production of Th2 cytokines which significantly elevates Interleukin 5. The hAEC secrete transforming growth factor-beta (TGF-ß), a T cell growth inhibitor and a powerful immunosuppressive molecule (40). The hAEC also secrete other immunosuppressive factors including several interleukins (40, 41). Other immunoinhibitory molecules were reported to be expressed by hAEC, including CD59 and Fas ligand (FasL) (42, 43). Fas ligand is a peptide that plays an important role in immune modulation and limits the host immune response by binding to the Fas receptor on activated host immune cells leading to apoptosis (43). The hAEC express CD59 on their surface that prevents the lysis of the hAEC by inhibiting the host complement system. CD59 prevents the formation of the C9 polymerization complex, which is required for formation of the complement membrane attack complex (42).

## 6.5 Hepatic Differentiation of hAEC

The hAEC has several characteristics that makes them stand out among the other sources of stem cells available in the field of regenerative medicine. They are multipotent, have immunosuppressive properties, and are non-tumorigenic.

It has also been reported that hAEC can differentiate into hepatocyte-like cells under certain conditions (18, 25, 44). hAEC express significant levels of SOX-2, OCT-4, and NANOG and these genes diminish at the end of hepatic differentiation procedures and the cells begin to express mature hepatic genes. Another study showed that these cells can differentiate *in vivo* to cells with characteristics of mature hepatocytes. Importantly, amnion epithelial cells do not fuse with the host parenchymal hepatocytes when transplanted as determined by histological analysis (26). The cells were further analyzed by RT-qPCR to confirm the expression of albumin, cytochrome P450 and other liver genes (26).

# 7 CRYOPRESERVATION

Cryopreservation of cells is necessary to preserve structurally intact cells at very low temperatures for a long period of time (9, 45). Cryopreserved cells, available for immediate use, would be important for patients with ALF when an organ or hepatocytes are not available, and for repeated scheduled transplants for metabolic liver disorders. An added advantage of using cryopreserved cells is that each cell batch can be individually analyzed and characterized according to the release criteria established for the transplantation of the cells. Thus, a cell bank can be established containing only those cells that meet all the criteria for use in a clinical transplant. These characterization steps can be accomplished long before the cells are actually needed for a transplant procedure.

However, the cryopreservation procedure can result in the formation of intracellular ice crystal, which leads to dehydration of cells. This can lead to cell rupture, necrosis, and apoptosis. The optimization of the cryopreservation solution, the cryoprotectant and freezing rate can minimize the damage to the cells.

## 7.1 UW Solution

University of Wisconsin (UW) solution was developed during the late 1980s by Belzer and colleagues at University of Wisconsin for the cold storage and transport of organs prior to transplantation. Later, UW solution was adopted for the cryopreservation of hepatocytes (9,12) and later, (46). Besides its effectiveness, an advantage of this solution for a clinical study is that the solution is produced and sold as a pharmaceutical-grade solution, which is approved for use on cells, which will later be transplanted into patients. The efficacy of the cryopreservation procedure depends not only on the cryopreservation solution but also on the nature of the cells being cryopreserved. Simple cells with few organelles such as fibroblasts or tumor cells generally cryopreserve easily, while complex cells such as hepatocytes, with many different organelles, all which might freeze at different rates, are generally more sensitive to cryopreservation procedures. Successful cryopreservation also depends on the cryoprotectants used, the freezing rate and thawing procedures. There are other commercially available cryosolutions, including cryostore, stem cell banker are also used for cryopreservation (47, 48). The Institut Georges Lopez (IGL) solution is also commercially available cryosolution, which is similar to UW solution although it is lower in potassium and viscosity (49).

## 7.2 Cryoprotectant

Cryoprotectants are generally used to protect the cells from freezing damage. Naturally, amphibians and fish in Arctic and Antarctic regions produce anti-freeze compounds in their body (mainly sugars) to reduce the damage caused by freezing. Mammalian cells are not suited for direct cryopreservation. When the cells are cryopreserved, they tend to form intracellular ice crystals. The ice formation is avoided and flexibility is maintained by the cryoprotectant reagents (50, 51). Dimethyl sulfoxide (DMSO) is as a commonly used and accepted cryoprotectant reagent. The final concentration of 10% DMSO is generally used in protocols (50, 51). In addition to DMSO, there are various cryoprotectants being used for long-term storage for liver cells. Glycerol, fetal bovine serum, sucrose is also used as a cryoprotectant along with cryopreservation solutions without DMSO (52).

## 7.3 Freezing Rate

The cells are cryopreserved in a controlled rate freezer instead of rapid freezing. The temperature is lowered to -80°C at a controlled rate of -1°C per min. Subsequently, cells are moved to -150°C (vapor phase of liquid nitrogen storage tank).

# 8 AMNION EPITHELIAL CELLS IN PRECLINICAL STUDIES

Preclinical studies have to be conducted to take hAEC further as a real tool for cell therapy. Preliminary studies on animal models with active immune system help us to expand the knowledge on hAEC and to understand the characteristics of the cells *in vivo*, handling of the cells for transplantation and potential of the cells.

The hAEC have gained recognition as an alternative to whole organ transplantation in acute liver failure, chronic liver disease and metabolic disorders (28, 53). Preclinical studies have been conducted with mouse models and showed hAEC are effective and likely safe for clinical transplant. Animal models that mimic conditions in human metabolic liver diseases including Maple syrup urine disease (MSUD), Phenylketonuria (PKU) and acute liver failure were corrected by hAEC transplants (28, 54).

## 8.1 Site of Administration

One of the important strategies in cell therapy of liver disease is to deliver the cells to the liver with minimal leakage to other organs. The most common site for transplanting cells are the spleen and liver. However, the transplantation site is chosen based on the liver architecture. The route of administration must be carefully considered when developing cell therapies. For hepatocyte transplants, an intraportal route is preferred for clinical transplants (55). This can be accessed by puncturing the portal vein with a catheter. In younger patients the liver can also be accessed through the umbilical vein (56). When the liver architecture is altered as in the patients with cirrhosis, hepatocyte transplantation may lead to embolization of the portal veins and portal hypertension. There are alternative sites that can be used for cirrhotic patients such as the splenic pulp or splenic artery, or the peritoneal cavity (9, 12, 57, 58). In animal models, the intraportal route is preferred via spleen to achieve better biodistribution of the cells in the liver. When there is a need for multiple transplants of cells, cells can be directly transplanted to the liver.

## 8.2 Bio-Distribution

To improve the efficiency of cell therapy and to minimize side effects, it is important to optimize the route of administration of cells and confirm the transplanted cells reach the target organ, in this case, the liver. There is a need for reliable, non-invasive methods to evaluate the bio-distribution, migration, and engraftment of the cells after transplantation. Non-invasive imaging techniques are designed for tracking the transplanted cells and to determine their bio-distribution. Bio-distribution studies were performed with different cell types including embryonic stem cells,

hepatocytes and mesenchymal stromal cells with different reporter markers such as near infra-red dye (NiR), 111-Indium labeling and magnetic resonance imaging (MRI) (59-64). For better understanding of hAEC, a bio-distribution study was published by our group recently (65). The majority of hAEC transplanted reached the liver when they were infused into the portal vein via the spleen. Migration of cells is negligible to other organs. To move forward with preclinical studies, mouse models with metabolic liver diseases were used to investigate the efficacy of hAEC transplants to correct the symptoms of the genetic disease.

# 9 ANIMAL MODELS TREATED WITH HAEC

## 9.1 Phenylketonuria (PKU)

The deficiency of phenylalanine hydroxylase (*PAH*) occurs due to mutation in the *PAH* gene. It is an autosomal recessive disorder, i.e. parents are frequently the carriers of the disease, although they usually do not suffer from the mutation. With *de novo* mutations, or when both the parents transmit the mutant gene, it can result in PKU in the child. The level of the deficiency depend on the nature of the mutation, which may result in serious PKU or mild hyperphenylalaninemia. The prevalence of PKU is 1 in 10,000 in Europe and a higher incidence found in turkey with 1 in 4,000 births and less common in African and Asian populations (https://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=EN&Expert=716).

The mutation results in the inability to metabolize phenylalanine to tyrosine. The lack of *PAH* expression, or the production of mutant protein results in high phenylalanine levels in the blood and brain that cause neurological problems (66). In contrast, tyrosine is not produced significantly and it is required for proper neural development since it is an important enzyme for synthesis of neural transmitters (Figure 8).

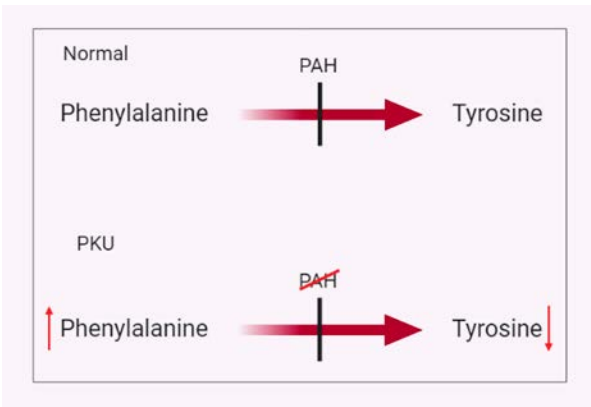


***Figure 8.*** *Pictorial representation of phenylalanine built up due to the PAH deficiency and in- contrast, insufficient tyrosine synthesis. Created with Biorender.com*

There is a mouse model for PKU that carries the same mutation as many human PKU patients, and the affected mice show many of the same symptoms of the disease as the PKU patients. In our studies, the pups were transplanted with previously cryopreserved hAEC directly into the liver. There was no immunosuppression provided when transplanting hAEC even though the mouse has a normal immune

system. This is one of the major advantages of hAEC cell therapy since with other cells, rejection is a major hindrance. When PKU mice were transplanted with hAEC there was a significant correction of the phenylalanine levels in the blood and brain. The mice were kept under observation for one hundred days without any sign of rejection or inflammation. The transplants of hAEC reduced brain phenylalanine to levels that are not significantly different from un-affected, wild-type animals (28). Organs were harvested for further analysis of hAEC. Gene expression profiling showed that hAEC expressed all 63 genes examined at levels that are expressed in mature liver cells.

## 9.2 Maple Syrup Urine Disease (MSUD)

Another severe metabolic liver disease, MSUD, is an autosomal recessive disorder of amino acid metabolism. It is estimated around 1 in 185,000 infants worldwide are affected with this metabolic disorder. This metabolic disorder gets its name because of the affected infants' urine smell with a distinct sweet odor. The disease is caused by the inability to metabolize branched-chain amino acids and is characterized by the accumulation of branched-chain amino acids (BCAA) in the blood and brain. Untreated individuals can experience neurological abnormalities, seizures, and ultimately, death (67). Currently, the disease is treated primarily with lifelong diet control with BCAA restriction.

MSUD results due to deficiency in the branched-chain-αketo acid dehydrogenase (BCKDH) complex. This mutation results in inability to process branched-chain-αketo acid (BCKA). This BCKA is derived from BCAA; leucine, isoleucine, and valine, respectively. The accumulation of BCAA is depended on the level of BCKDH deficiency which results in sweet maple syrup odor in the urine (53).

Animal models are useful to understand and explore the mechanism of disease and for preclinical studies. A mouse model was developed with a partial knockout of BCKDH. Our group previously reported the correction of MSUD by hepatocyte transplantation and extended these studies to include hAEC transplantation as a substitute for hepatocytes in these procedures. As with the transplantation of authentic hepatocytes, hAEC transplants resulted in increased survival of the animals as well as the correction of the BCAA in serum and brain (54, 68).

## 9.3 Acute Liver Failure

Acute liver failure is severe and rapidly progressing liver failure that is frequently treated by liver transplantation. The primary problem in ALF is the absence of parenchymal hepatocytes and this cell mass is need to support the metabolic requirements until the liver regenerates or a donor liver is available for transplant. Here, hAEC can provide an exogenous source of cells that can provide immune

modulation and ultimately, hepatic function to support the host liver and correct liver failure. Once the native liver starts to regenerate, OLT might not be needed and hAEC therapy can be done without immunosuppression.

Our group has reported earlier, hAEC can rescue the mouse model of ALF. A reproducible mouse model of ALF was generated by injecting a dose of d-galactosamine (d - gal) and that is 100% fatal to the mice if they were untreated. Mice were infused with 2 million hAEC via spleen 6h after the d-gal treatment (28).

All mice that received only d-gal died within 72h, while 100% of the mice that received hAEC transplants survived the d-gal treatment (28). These preclinical studies cited above demonstrate the efficacy of hAEC in the treatment of acute and chronic, metabolic liver disease.

# 10 AMNION EPITHELIAL CELL THERAPY OF OTHER TISSUES

Apart from liver, hAEC is also used for several diseases affecting different organs. In a mouse model, lung injury was induced with bleomycin and hAEC transplants reduced the inflammation and severity of disease (69). Recently, a human trial with hAEC was successfully performed in six premature babies with Bronchopulmonary Dysplasia (BPD) (70). Lung fibrosis was successfully prevented and the hAEC were well tolerated and there was no evidence of tumor formation and no adverse effects were observed in any of the babies. In other studies, hAEC were shown to differentiate to neuronal-like cells, synthesize and release catecholamine; a neural transmitter that serves in transferring signals between neurons (71). This makes hAEC a potential candidate for neural related disease such as Parkinson disease (71) (72).

Diabetes mellitus has become a serious threat to human health. Replacing the lost Beta cells with functional insulin-producing Beta cells is a novel therapy that could provide a cure for patients with Diabetes mellitus. It has been reported, that when a patient with type I Diabetes mellitus was treated with amniotic cells, the patient remained independent of insulin intake for 6.2 months and later, insulin requirements were readjusted to 8 IU/day post-transplantation compared to 38 IU/day, pre-transplant (73). It was also reported, that hAEC have the potential for wound healing (74), and the correction of heart disease (75), stroke (76) multiple sclerosis (40) and many other diseases (Figure 9).

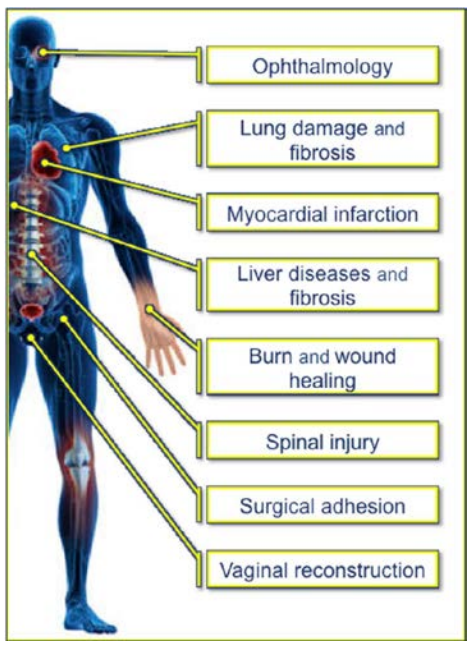


***Figure 9.*** *Pictorial representation of amnion tissue and cells used to treat the medical problem. S.C. Strom and R.Gramignoli, Human Immunology 77 (2016) 734-739*

# 11 ANIMAL MODELS

Animal models are used to study the development and progression of disease and are used to test new medications and treatments before they are given to humans. There are multiple factors that affect liver function and billions of dollars are spent across the world in medical care to treat liver-related diseases. There are large differences in metabolic pathways involved in xenobiotic metabolism and excretion between the commonly used animal models and humans. Experiments with mouse models may not faithfully predict what occurs in humans. Recent developments in animal models have resulted in creating mouse models with genetic alterations that result in damage and loss of native hepatocytes. Under appropriate conditions, the loss of the mouse hepatocytes creates a strong liver regeneration response that supports the complete replacement of the mouse hepatocytes with human hepatocytes in the liver of these mice.

## 11.1 Humanized Mice

To address these issues, several groups tried to make chimeric mice with 'Humanized liver' by engrafting and expanding primary human hepatocytes in rodents. Markus Grompe, Strom and co-workers developed a model with a *Fah* knockout mouse that was crossed with *Rag2*$^{-/-}$ (recombinant activation gene 1) and *Il2rg*$^{-/-}$ (interleukin receptor 2 subunit gamma) mice that result in triple mutants *Fah/Rag2/Il2rg* (FRG) mice. The mouse is profoundly immunodeficient and will accept the transplantation of cells from different species, including human (77). Also, the murine *Fah* mutation is equivalent to the severe metabolic liver disease, Hereditary Tryosinemia type 1( HT1). Like human HT1 patients, the mice will not develop liver disease while they are maintained on the protective drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1, 3-cyclohexanedione (NTBC). However, when NTBC is withdrawn, the mice go into liver failure and can be rescued by the transplantation of FAH-proficient human hepatocytes (77). In this model, the mouse hepatocytes can be nearly entirely replaced as the mouse liver becomes repopulated with human hepatocytes. Later studies incorporated the non-obese diabetic (NOD) mouse background into the FRG strain to generate the FRGN mouse. The additional presence of the NOD background enables a greater acceptance of human cells as compared to the normal FRG strain (78). The liver-humanized mice are useful models for drug metabolism and excretion and toxicity studies (79, 80). The FRGN model is also useful for the bio-distribution studies since immunosuppressive drugs are not required even when the animals receive xenotransplats of human cells, such as hAEC.

# 12 AIM AND SIGNIFICANCE

The overall aim of this thesis is perform basic, preclinical studies of the safety and efficacy of hAEC transplants to treat liver diseases and to set a basis for more targeted studies to translate human amnion epithelial cells to the clinic. Amnion-derived cells have been characterized and validated as a homogenous population after isolation procedure, while *in vivo* studies demonstrated their ability to differentiate into functional hepatocyte-like cells. The hAEC could have a substantial impact on the cell therapy field.

The objective of individual papers are as follows:

**PAPER 1:**

- To standardize and optimize a cell isolation protocol for manufacturing hAEC under GMP-like conditions.

**PAPER 2:**

- To validate the cryogenic procedures for primary human amnion epithelial cells.

**PAPER 3:**

- To evaluate a hAEC-based treatment of the congenital liver disorder (PKU) in a relevant mouse model.

**PAPER 4:**

- To generate a liver-humanized mouse model for carbamoyl phosphate synthetase I deficiency and determine if it faithfully recreates the human disease.

**PAPER 5:**

- To validate efficient and safe routes for infusion of hAEC into the liver.

# 13 METHODOLOGY

## 13.1 Primary cells

### 13.1.1 Murine And Human Hepatocyte Isolation

The mouse hepatocyte isolation was performed by *in situ* perfusion of the liver by a three-step collagenase perfusion protocol. Briefly, the first solution consist of Hank's Balanced Salt Solution (HBSS) supplemented with 1 mM ethylene glycol tetraacetic acid (EGTA), a calcium chelator which helps in disrupting the intracellular connections by removing the calcium. A second solution of HBSS is supplemented with calcium since it is required for collagenase to function and to remove the EGTA from the liver. Finally, a third solution of HBSS containing collagenase type 2 (1 mg/mL), is used to disrupt the extracellular matrix.

A retrograde perfusion of the liver is accomplished by inserting a catheter into the inferior vena cava with additional occlusion of the upper vena cava with a clip. A transection of the portal vein allows perfusate to escape the liver. Perfusion solutions were kept at 37°C in a water bath. Digestion of the liver takes about 8 to 12 min depending on the mice. The digested liver was transported to the lab in cold HBSS on wet ice and chopped with a scissors in a sterile beaker to release cells. The cells were centrifuged at 90 g for 5 min with two additional washes and centrifuged at 4 °C. The cells were counted and cryopreserved using UW solution supplemented with 10% DMSO.

All human tissues were collected with informed consent following institutional and ethical guidelines (ethical protocol 2014/1561-32). Organ donors and explanted tissues were tested for hepatitis viruses B and C, and human immunodeficiency virus (HIV) and resulted negative. All human hepatocytes were isolated as previously described (81), and immediately after isolation were seeded in culture plates or cryogenically preserved using University of Wisconsin solution (BEL GEN 1000, Lissieu, France) supplemented with 10% DMSO (Sigma-Aldrich, St. Louis, MO, USA). Hepatocytes were thawed on the day of transplantation in a 37 °C water bath until the ice is barely visible and cells are diluted with 10 volumes of cold Williams medium E supplemented with 10% calf serum and centrifuged at 90 g for 5 min at 4 °C. The cell pellet was suspended in cold plasmalyte (Baxter, Norfolk, UK) for transplantation.

### 13.1.2 Murine Transplantation Procedures

For intra-splenic transplants, mice were anesthetized using isoflurane (Baxter, Norfolk, UK) and a small incision was made on the left abdominal region and the tip of the spleen was exposed. The cells were infused slowly into the splenic parenchyma. Cell leakage or overflow of cells was prevented by placing the

cotton-tipped applicator on the injected site for a few seconds. The spleen was placed back in the abdominal cavity and the incision was closed with absorbable sutures (Ethicon, Diegen, Belgium). For tail vein infusions, mice were placed in a restrainer and cells were infused by a syringe with a 29 gauge needle. All transplant events required an infusion procedure not longer than 40 sec. Transplants consisted of one million (viable) cells re-suspended in 200µl plasmalyte solution with 15 units/mL heparin (Orifarm AB, Stockholm Sweden)

### 13.1.3 Human Amnion Epithelial Cells

Human Amnion epithelial cells were obtained by enzymatic digestion of human amnion membranes from full term placentae. We used human placentae procured only from uncomplicated full-term cesarean procedures performed at Karolinska University Hospital, Huddinge. Placenta are procured after signed consent from the mother according to ethical protocol and permit number 2015/419-34/4. Tissue is collected after cesarean sections from pre-screened healthy mothers for sterility and safety concerns. The protocol for hAEC isolation is based on mechano-enzymatic procedure as previously described (82). Briefly, the amnion membrane was surgically removed from the surface of the placenta and washed multiple time with Ringers solution (Baxter, Sweden) to remove the blood from the membrane. A final wash in Saline solution (Fresenius kabi, Sevres, France) is performed to re-equilibrate pH to 7.4. The amnion membrane is equally distributed in 50 ml falcon tubes (approximately 2-3 gr of wet tissue per tube) and resuspended in TrypLE 10x solution (Life tech, Paisley, UK). All the tubes were transferred to a temperature-controlled rotator (IncubatorGeni, Bohemia, New York), and left at 37 °C in rotation at 35 rpm for 30 min to specifically remove epithelial cells. The remaining tissue is discharged and the dispersed epithelial cells collected and washed at least 2 times by centrifugation at 400 g for 5 min at 4 °C. The cell pellet was resuspended in saline solution and filtered through 100 µm cell strainer to remove cell clumps and remaining small membrane fragments.

### 13.1.4 *In Vivo* Cell Tracker

Cryopreserved hAEC were thawed quickly in a water bath at 37 °C, centrifuged at 450 x g for 5 min and resuspended in plasmalyte solution (Baxter, Norfolk, UK). Cells were counted with a Bürker chamber and viability was quantified by Trypan Blue exclusion (Sigma-Aldrich). Cells were directly labeled with the lipophilic DiR dye (1,1'-dioctadecyltetramethyl indotricarbocyanine Iodide; (product no: 125964, Perkin Elmer, Waltham, USA), and cell viability was measured again (by Trypan Blue exclusion). DiR dye has a near infra-red emission wavelength with the excitation/emission range from 748/780nm. The staining solution was prepared at a final concentration of 5 µM dye in plasmalyte solution. The hAEC were suspended in the staining solution and incubated at 37 °C for 30 min with

mild rocking. Later, cells were sedimented at 450 g for 5 min and washed three times with cold plasmalyte to remove unbound dye. Each animal received one million hAEC resuspended in 200 µl plasmalyte, supplemented with 15 units(U)/ml heparin (Orifarm AB, Stockholm, Sweden), and transplanted via tail-vein or directly into the parenchyma of the spleen.

### 13.1.5 *In Vivo* Imaging System (IVIS)

Anesthetized mice were placed into the IVIS chamber and images were captured using the IVIS spectrum camera (Perkin Elmer) at 1, 3 and 24 h. post cell transplantation. Background level was quantified by transplanting unlabeled-hAEC in one mouse per group. At the end of total body scan, 24 h. post-infusion, mice were euthanized, and organs were removed and placed into the IVIS chamber for *ex vivo* imaging. Both total blood and plasma samples were collected and measured at 24 h. post-transplant. Plasma samples were collected in a heparinized, Microvette® 500 K2E tube (product no: 20.1339.100, Sarstedt, Germany) and centrifuged at 450 g for 5 min. Blood and plasma samples were imaged with IVIS to quantify signal from cells in the circulation by seeding 200µl of total blood or plasma in a black 96 well plate and fluorescence was read with the IVIS spectrum camera.

### 13.1.6 Blood and Tissue Sampling

Starting from the 5$^{th}$ week after human hepatocyte transplantation, the levels of circulating human albumin were monitored to estimate the level of repopulation of the mouse liver with human hepatocytes. Blood samples were collected from mice twice a month. The mice were placed in a restrainer and blood samples were collected from the tail vein using 27 gauge needle.

When the experiments were complete the mice were anaesthetised using isoflurane. Blood and internal organs (liver, lungs, spleen, kidneys) were harvested and portions were immediately flash frozen in liquid nitrogen and stored at -80 °C. The representative tissue was fixed in 4% formalin for 48 h. and embedded in paraffin. The tissue sections were sectioned to 4µm thickness and mounted on a Superfrost® Plus glass slides (VWR, Leuven, Belgium).

## 13.2 Immunostaining techniques

### 13.2.1 Fluorescence-Activated Cell Sorting (FACS) Analysis

The evaluation of surface markers expression was performed by flow cytometry. Flow cytometry was performed on both freshly isolated and cryopreserved hAEC. The hAEC were incubated for 30 min at 4°C in 100µl ice-cold PBS with specific antibodies (listed in Table below).

**Table 1.** List of antibodies used for flow cytometry.

| Antibody | Dye | Clone | *Ig* | Host | Diluition | Company | cat. Number |
|---|---|---|---|---|---|---|---|
| CD326 (EpCAM) | APC-conjugated | HEA-125 | IgG1 | Mouse | 1:10 | Miltenyi | 130-091-254 |
| CD105 (endoglin) | PE-conjugated | 266 | IgG1 | Mouse | 1:20 | BD | 560839 |
| CD49f (alpha6-Integrin) | PE-conjugated | GoH3 | IgG2a | Rat | 1:5 | BD | 555736 |
| CD45 | FITC-conjugated | T29/33 | IgG1 | Mouse | 1:10 | Dako | F0861 |
| CD44 (H-CAM) | FITC-conjugated | G44-26 | IgG2b | Mouse | 1:5 | BD | 560977 |
| CD31 (PECAM-1) | Alexa647-conjugated | WM59 | IgG1 | Mouse | 1:20 | BD | 561654 |

Cells were either stained with single or multiple markers simultaneously, and signal discriminated by different detectors. Isotype controls were used to measure the level of non-specific background signal caused by primary antibodies. After primary antibody incubation, cells were washed to remove the unlabeled antibodies and fixed with 2% BD™ stabilizing fixative (BD biosciences, San Jose, CA, USA) for 10 min at room temperature. The cells were centrifuged, resuspended in ice-cold PBS (300µl) and analyzed using BDCanto and FlowJo™_v10 software.

### 13.2.2 Immunohistochemistry

Liver samples from mice were stained with human-specific antibodies; CPS1 (1:25) (Dako, Glostruo, Denmark), OTC (1:400) (Sigma, St. Louis, MO, USA) and/or CK8/18 (1:100) (ThermoFisher, Rockford, IL, USA) and tissues were counterstained with hematoxylin and eosin (HE) to quantify the level of engrafted cells.

## 13.3 Molecular techniques

### 13.3.1 DNA Analysis

To quantify human cells in different organs, murine tissues were crushed into a fine powder under liquid nitrogen, and 8 aliquots from each organ were collected. Genomic DNA was isolated from tissue samples using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Total human DNA content was estimated using quantitative polymerase chain reaction (qPCR) with human-specific AluYb8 sequence (forward:5'CGAGGCGGGTGGATCATGAGGT3', reverse: 5'TCTGTCGCCCAGGCCGGACT3', Invitrogen). Linearity and resolution limits were determined by diluting hAEC DNA in murine DNA at concentrations of: 30%, 10%, 3%, 1%, 0.3%, 0.1%, 0.03% and 0.01%. Mouse liver DNA was used as a negative control. The sensitivity of the assay is such that one human cell in 10.000 mouse cells could be detected.

### 13.3.2 Elisa

From each mouse, 2 μL blood was collected, diluted in 198 μL diluent and assayed using the Quantitative Human Albumin ELISA Quantitation Kit (Bethyl Laboratory, TX, United States) according to the manufacturer's protocol. Multiple measurements were performed on each sample, at different dilutions (1: 100-100.000), in order to quantify the level of "humanization". It is estimated that 1 mg/ml of circulating human albumin represents a 20% of repopulation with human hepatocytes (77, 83). A fresh standard curve (ranging from 15 to 200 ng/mL) made with human reference serum RS10-110-4 (Bethyl laboratory Inc., TX, USA) was included in each analysis.

### 13.3.3 Quantitative Real Time PCR

The RNA was isolated by lysing cells using Trizol™ solution (Thermo Fisher, Waltham, MA, USA) and the tissue samples were homogenized using iron beads using tissue homogenizer and RNA was isolated according to the manufacturer's instructions. Total RNA was converted to complementary DNA using a high capacity cDNA kit (Thermo Fisher, IL, USA). The DNA was isolated from cells and tissue using DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Gene expression was assessed using TaqMan™ assays (Thermo Fisher, Waltham, MA, USA). Few TaqMan™ assays were custom made to be specific to human and not to cross-react with mouse cells.

**Table 2.** TaqMan assays.

| No. | Gene | Assay | No. | Gene | Assay |
|---|---|---|---|---|---|
| 1 | PPIA | Hs99999904_m1 | 34 | NTCP | Hs00914889_m1 |
| 2 | GAPDH | Hs03929097_g1 | 35 | HNF1a | Hs00167041_m1 |
| 3 | CYP 3A4 | Hs00430021_m1 | 36 | HNF1b | Hs00172123_m1 |
| 4 | CYP 3A7 | Hs00426361_m1 | 37 | HNF3a | Hs04187555_m1 |
| 5 | CYP 1A1 | Hs00153120_m1 | 38 | HNF3b | Hs00232764_m1 |
| 6 | CYP 1A2 | Hs01070374_m1 | 39 | HNF4a | Hs00230853_m1 |
| 7 | CYP 2C9 | Hs00426397_m1 | 40 | HNF6 | Hs00413554_m1 |
| 8 | CYP 2D6 | Hs02576167_m1 | 41 | HIF1A | Hs00153153_m1 |
| 9 | CYP 2C8 | Hs00258314_m1 | 42 | DLK1 | Hs00171584_m1 |
| 10 | CYP 2C19 | Hs00426380_m1 | 43 | NANOG | Hs04260366_g1 |
| 11 | CYP2B6 | Hs03044634_m1 | 44 | SOX2 | Hs01053049_s1 |
| 12 | UGT1A1 | Hs02511055_s1 | 45 | SOX17 | Hs00751752_s1 |
| 13 | UGT1A6 | Hs01592477_m1 | 46 | OCT4 | Hs00742896_s1 |
| 14 | UGT1A9 | Hs02516855_sH | 47 | CK7 | Hs01115174_mH |
| 15 | UGT2B7 | Hs00426592_m1 | 48 | CK8 | Hs02339474_g1 |
| 16 | UGT2B17 | Hs00854486_sH | 49 | CK18 | Hs01653110_s1 |
| 17 | A1AT | Hs01097800_m1 | 50 | CK19 | Hs00761767_s1 |
| 18 | OTC | Hs00166892_m1 | 51 | ASGR1 | Hs00155881_m1 |
| 19 | CPS1 | Hs00157048_m1 | 52 | MET | Hs01565582_g1 |
| 20 | PAH | Hs00609359_m1 | 53 | CYP7A1 | Hs00167982_m1 |
| 21 | ALB | Hs00609411_m1 | 54 | UGT2B10 | Hs02556282_s1 |
| 22 | AFP | Hs00173490_m1 | 55 | LXRα | Hs00172885_m1 |
| 23 | PXR | Hs01114267_m1 | 56 | LXRβ | Hs01027215_g1 |
| 24 | CAR | Hs00901570_g1 | 57 | CYP7B1 | Hs00191385_m1 |
| 25 | AHR | Hs00169233_m1 | 58 | SOX9 | Hs01001343_g1 |
| 26 | FXR | Hs01026590_m1 | 59 | PPARα | Hs00947536_m1 |
| 27 | P-gp | Hs00184500_m1 | 60 | PPARγ | Hs01115513_m1 |
| 28 | MDR3 | Hs00240956_m1 | 61 | WNT1 | Hs01011247_m1 |
| 29 | BSEP | Hs00184824_m1 | 62 | catenin, β | Hs00355049_m1 |
| 30 | MRP2 | Hs00166123_m1 | 63 | Glutamine synthase | Hs00365928_g1 |
| 31 | MRP3 | Hs00978473_m1 | 64 | Glucocorticoid receptor | Hs00353740_m1 |
| 32 | MRP4 | Hs00988717_m1 | | | |
| 33 | BCRP | Hs00184979_m1 | | | |

# 14 AMMONIA CHALLENGE

The CPS1-D and CPS1 proficient liver humanized mice were characterized by their ability to metabolize ammonia. The ammonia challenge was performed on mice by an intraperitoneal injection of [$^{15}$N] ammonium chloride (product number 299251, >99% pure, Sigma-Aldrich, St. Louis, MO, USA) 4 mmol/kg of body weight. The level of ammonia was measured before and 30 min post-injection of ammonia using an Arkray pocket chem (Arkray, AT, Netherlands) according to the manufacturer's instruction.

# 15 STATISTICAL ANALYSIS

In all studies, data sets were compared by Mann-Whitney non-parametric tests, since the collected data were not normally distributed. Results are represented as histograms showing mean ± SD, or box and whisker plots showing median, 25- and 75-percentiles. P-value < 0.05 was considered as significant (*P < 0.05; **P < 0.001; ***P < 0.0001). Data were analyzed with GraphPad Prism software, version 6 (GraphPad Software Inc., San Diego, CA, USA).

# 16 RESULTS AND DISCUSSION

## 16.1 Paper 1

**Isolation of Human Amnion Epithelial Cells According To Current Good Manufacturing Procedures.**

The precise requirement for banking of any cell product for clinical use may vary, but it is highly recommended that all reagents used in the isolation procedure do not contain and come from animal origin. The practice of stem cell bio-banking has been growing in recent years due to supportive results in their use for cell therapies. Several countries have established cell banking practice to preserve stem cells and facilitate their research and clinical application (84).

Although the published study was conducted under regular laboratory conditions, and are thus, not "According to Current Good Manufacturing Procedures" as the title suggests, the reagents and techniques were standardized so that they could be translated to protocols in a GMP-level facility. We make no claim of isolating these cells under GMP conditions, just the working out of the techniques with GMP grade reagents to facilitate a quick translation of the technology to a GMP level protocol. In this study, we have established a protocol to isolate amnion epithelial cells from the placenta with minimal contamination of other cell types. hAEC suspensions has been previously transplanted in mouse models of metabolic liver diseases, establishing the safety and efficacy of hAEC (18, 28, 54). Based on our previous preclinical and clinical transplants using human hepatocytes, it is accepted that large numbers (billions) of donor cells may be required to correct metabolic diseases, and such large numbers of primary hAEC cannot be obtained from a single placenta. Combined, repeated hAEC infusions from multiple donors will most likely be required in clinical practice. Thus, hAEC isolation and bio-banking is an essential step to have a sufficient number of hAEC 'off-the-shelf' that can be transplanted "on-demand".

Amnion-derived cells are classified as Advanced Therapy Medicinal Product (ATMP) according to the European Union (EU) classification. Criteria for ATMPs can be found in Article 17 of Regulation (EC) No. 1394/2007. The aim of project 1 was the isolation of hAEC from the full-term placentae in near GMP conditions. Our protocol is based on minimal manipulation (enzymatic isolation and cryopreservation). A description of the isolation procedure is described in the methodology section (13.1.3). The workflow of the hAEC isolation procedure is represented in Figure 10.

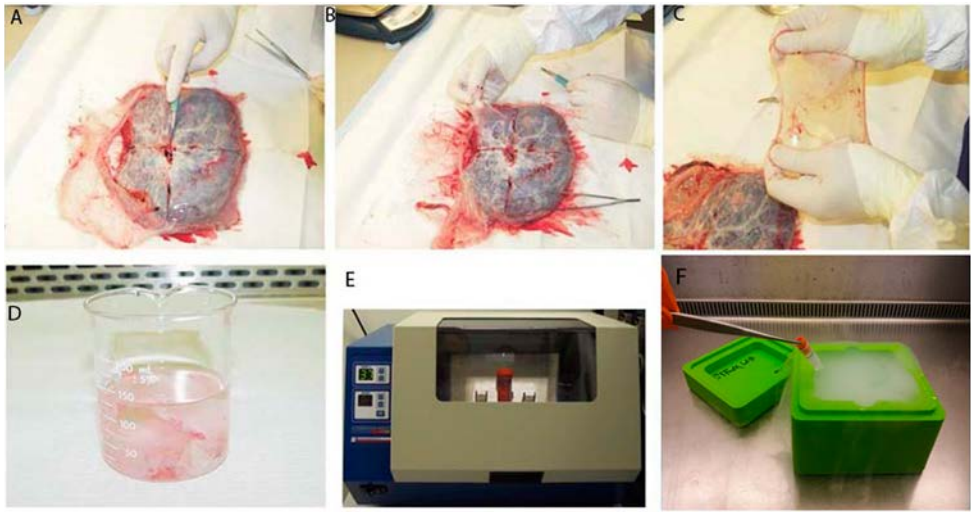


***Figure 10.*** *Amnion epithelial cell isolation from full-term human placenta. An X-shape incision is made on the placenta (A) and amnion membrane is manually peeled off (B and C). The amnion membrane is washed with Ringers (D) and digested using TrypLE solution at 37°C in continuous agitation (E). Resulting cells are resuspended in cryosolution, transferred in freezing box and later moved in liquid nitrogen vapors (F). R. Gramignoli et al., Curr. Protoc. Stem Cell Biol 2016. 37:1E.10.1-1E.10.13.*

The efficiency and selective isolation of hAEC have been confirmed by histological analysis, where the removal of epithelial cells was confirmed after enzymatic digestion.

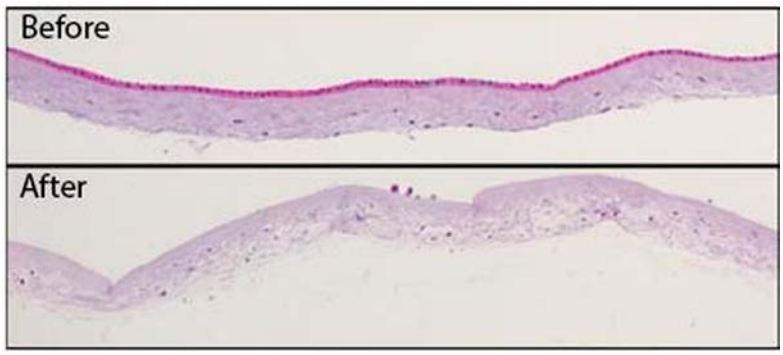


***Figure 11.*** *Amnion membrane before and after the hAEC isolation procedure. R. Gramignoli et al., Curr. Protoc. Stem Cell Biol 2016. 37:1E.10.1-1E.10.13.*

All the placenta tissues analyzed in the study have been collected at the Karolinska University Hospital (GYN/OB Department) with properly signed consent form and they are transported to the lab within an hour of delivery.

The release of the final hAEC product is based on tripartite components commonly required by International Agencies: sterility, viability, and identity. The presence of cell surface markers has been recognized as a method to validate hematopoietic and mesenchymal cells, thus we use this technology to identity amnion-derived cells. To validate hAEC products, we quantified expression of epithelial markers, such as CD326 and CD49f, by flow cytometry. Such analysis confirmed that the preparations showed minimal contamination of the epithelial cells with mesenchymal stromal cells (CD105 and CD44 positive cells) endothelial (CD31 positive) or hematopoietic cells (CD45 positive).

## 16.2 Paper 2

**Effect of cryopreservation on Human Amnion Epithelial Cells**

The long-term preservation (banking) of clinical-grade cell products, without compromising safety and efficacy, is essential to meet the required number of cells for transplantation. From the clinicians' point of view, the *sine qua non* conditions in cell therapy are, primarily, 'safety', then 'therapeutic efficiency' and finally 'availability/sufficient quantity'.

In our study, we have standardized a method for hAEC isolation and banking with GMP grade reagents. We validate hAEC batches by flow cytometry, based on the same surface markers we validated in paper 1, confirming marker expression before and after cryopreservation to evaluate the effects of cryopreservation on cells.

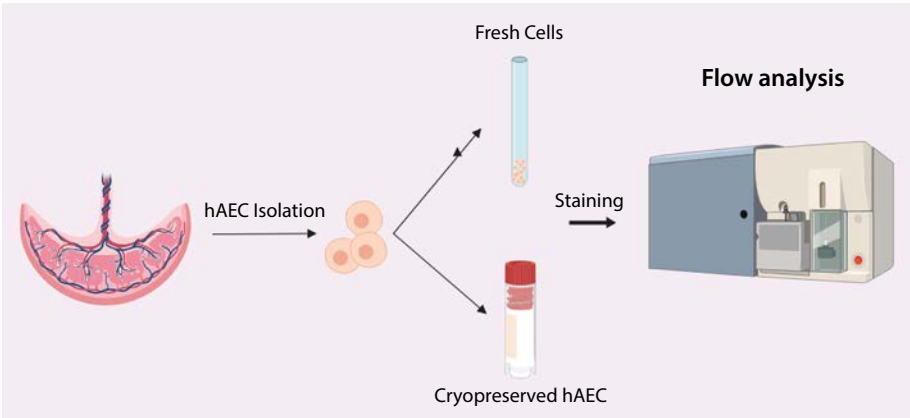


***Figure 12.*** *Project workflow. The hAEC were isolated from the placenta and characterized by flow analysis with fresh and cryopreserved cells. Created with Biorender.com*

Amnion epithelial cells were isolated as described in paper 1. In this study, hAEC were harvested from 14 different donors, cryopreserved and analyzed for purity using flow cytometry. Cryopreservation was performed using UW solution, supplemented with 10% DMSO. The density of 10 million cells per mL of cryopreservation media was chosen to freeze the hAEC. To confirm homogenicity, hAEC suspensions were stained for epithelial markers (CD326 (EpCAM) and CD49f (Integrin α-6 molecule), mesenchymal; CD105 (Endoglin molecule) and CD44, hematopoietic; CD45 and endothelial marker CD31. Cell viability was determined by Trypan blue exclusion at the time of isolation and after thawing.

Surface marker expression analyzed before the cryogenic procedure was not significantly altered after thawing, providing evidence that the same cells that were isolated, were recovered after freezing and thawing. We documented a decrease in cell viability of approximately 12% and up to a 35% decrease in cell recovery. We also monitored the presence of immunomodulatory molecules, such as non-canonical HLA-Ib and ecto-enzymes at the transcriptome level and by flow analysis. The expression of potent immunomodulatory molecules such as (HLA-G/-E/-F) are likely factors that explain the long term survival of hAEC and the ability to avoid immune recognition and rejection, in allogenic and even xenogeneic transplantation. We confirmed the low expression of polymorphic HLA-Ia as well as the lack of expression of HLA class II molecules.

We analyzed the ectoenzymes expressed by hAEC; CD39, CD38, and CD73, ectoenzymes that play vital roles in modulation of the host immune system (85). Along with the other immune-suppressive molecules, adenosine (ADO) has been identified as an important immunomodulator. Normally, ADO is produced via a canonical pathway; CD39, CD73 and an alternative pathway; CD38, CD203a/PC-1, CD73. An ecto-nucleoside triphosphate diphosphohydrolase (CD39) converts ATP to ADP and then into AMP and finally CD73 converts AMP into ADO. Alternatively, CD203a/PC-1 can convert ADP ribose or ATP to AMP and finally metabolize to ADO by CD73. The expression of these potent immunomodulatory ectoenzymes was present on all hAEC cases.

It is known that hAEC possess some degree of plasticity, likely due to the unique derivation of these cells prior to gastrulation and the specification of the three germ layers. The expression of characteristic stem cell genes, *NANOG, SOX2*, and *OCT4* were identified in all hAEC cases. *NANOG* and *SOX2* were expressed at 100 and 1000-fold different levels when compared to iPS cells, whereas *OCT4* expression was the same as iPS cells.

Cell migration, invasion, tissue remodeling, and proliferation are important steps that occur after cell transplantation. Important MMPs involved in cell mobilization are MMP 2 and 9 (86), and MMP 2 and 9 were expressed in all our hAEC cases.

Active MMPs are regulated, in part, by the tissue inhibitors of metalloproteinases (TIMPs). Maintaining the balance between MMPs and their inhibitors is critical for tissue remodeling. Finally, hAEC do not express telomerase reverse transcriptase (TERT), showing they are not immortal.

In conclusion, hAEC can be efficiently cryopreserved for clinical use without evidence of significant alteration of the surface receptors that are used to identify the cell population.

## 16.3 Paper 3

**hAEC transplantation as a treatment for Phenylketonuria**

Hepatocytes have always been the first choice of cells for transplantation to treat (congenital and acute) liver disease. An alternative source of cell needs to be identified as the availability of hepatocytes are not sufficient to meet all the demands for cell therapies. In paper 3, (currently reviewed and in revision based on the reviewer's comments for resubmission) we tested the hypothesis that hAEC can correct another inborn error of metabolism: PKU. Similarly to what we did in a previous study, where animals were rescued from another amino acid disorder (MSUD), we transplanted human amnion cells into an immunocompetent mouse with mutations in the gene that causes PKU (*Pah*). Our previous study with MSUD provided evidence of *in vivo* hepatic differentiation of hAEC based on correction of the genetic defect and expression of many other genes characteristic of mature hepatocytes, suggesting hAEC can engraft and differentiate to cells that express mature hepatic genes at levels that are sufficient to support genetic based metabolic liver diseases. All the more importantly, a xenotransplant of hAEC was performed on the MSUD mice, animals with a normal immune system, and the cells were accepted and were sustained, long-term in the mice without providing drug-immunosuppression.

PKU is a rare disease that affects male and females in equal numbers. PKU babies are normal when they are born and in the neonatal period. Later, symptoms start to appear such as, vomiting, lethargy, seizures and pale pigmentation develops in skin and hair due to the high levels of phenylalanine. Accumulation of phenylalanine also prevents the transport of amino acid across the blood-brain barrier. This results in disruption of amino acid synthesis in brain due to inhibition of synthesis of neurotransmitters, which leads to severe mental disabilities.

In this study, we used a *Pah* mutant mouse strain which shows symptoms commonly observed in human patients, including high level of phenylalanine in the blood. Based on our clinical evidence collected after 27 years of hepatocyte transplants, it is established that to correct most of the liver based genetic defects (excluding

hemophilias), we need to repopulate the donor liver with more that 10% donor cells to compensate for the missing hepatic gene function. Here, each mouse was injected with a total number of 15 million cells directly into the liver by multiple injections over the first 28 days. Cell transplantation was performed without providing immunosuppressive drugs to animals with a normal immune system.

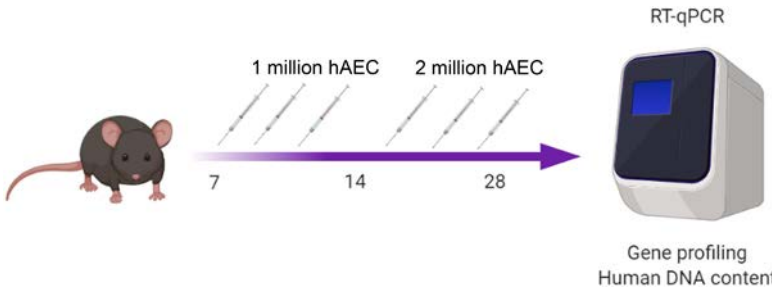


***Figure 13.*** *Project workflow. The hAEC were transplanted directly into the PKU mouse liver multiple times. The mice were euthanized on day 100 and analysed for gene profiling. Created with Biorender.com*

The DNA and RNA analysis 100 days post-transplantation revealed hAEC engraftment and expression of human PAH enzyme in the mouse liver. On average, livers were measured to contain 3.7% human DNA and a similar level of human PAH expression (4.6% of normal). There is a partial correction in serum PHE with reduction up to 60% when compared to the wild type mouse. Brain PHE was corrected to the level that it is not significantly different when compared to wild type mice. This implies hAEC transplants can provide substantial improvement of PHE levels in the brain and suggest that this therapy would provide disease correction. This data again shows the plasticity of hAEC and potential to become hepatocyte-like cells when transplanted to the liver. In addition, transcriptome analysis revealed that hepatic liver genes, commonly expressed in mature hepatocytes, were expressed at similar levels in hAEC post-transplant. Pre-transplant, hAEC do not express hepatic genes (including *PAH*) except for very low levels of CPS1 and UGT1A9, supporting the hypothesis of *in vivo* hAEC maturation into hepatocyte-like cells when they are transplanted into the liver. Apart from PAH expression, we measured mature liver like expression of other enzyme genes such as OTC, A1AT, CPS1, and BSEP, supporting the hypothesis that hAEC transplants could be effective in patients with these deficiencies.

Finally, we never observed neoplastic transformation of the cells post-transplant, consistent with previous studies (18, 26).

In summary, the efficacy of hAEC transplants was demonstrated in a mouse model of where injection of hAEC significantly improved the PHE levels. The xeno-transplant was performed in an immunocompetent mouse, supporting our hypothesis that hAEC can be transplanted without immunosuppressive drugs.

## 16.4 Paper 4

**A liver-humanized mouse model for carbamoyl phosphate synthetase 1-deficiency (CPS1-D)**

Animal models are usually used as a surrogate of human biology. Small animals are used and due to their small size, they can be handled easily and can be genetically manipulated. A major barrier in translating the preclinical data to the clinic is the biological difference between man and mouse. A notable example was Fialuridine (FIAU), which was used to treat HBV infection. Preclinical studies in mice showed no toxicity, however, when it was used on human patients, it resulted in severe liver injury (87).

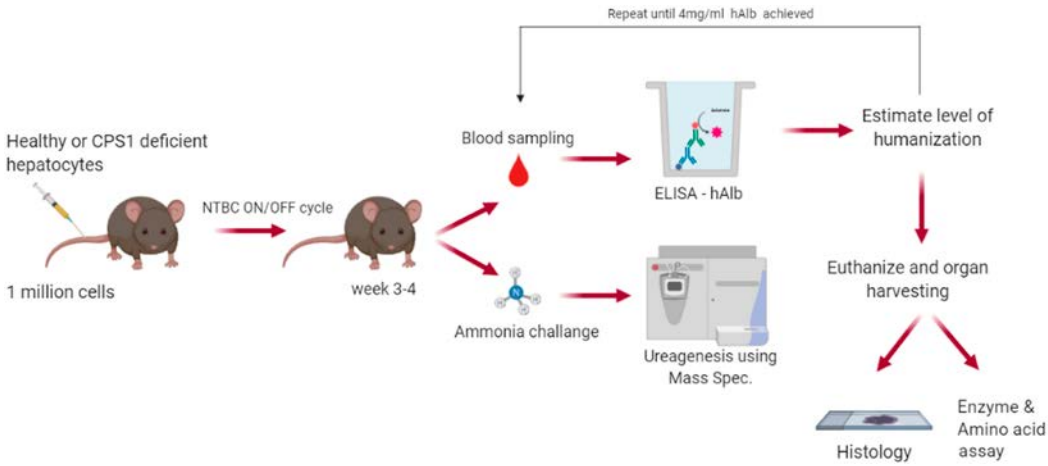


***Figure 14.*** *Project workflow. The FRGN mice were transplanted with CPS1-D or healthy hepatocytes. The blood sampling was performed to estimate the level of humanization using ELISA and ammonia challenge was performed to analyse the ammonia metabolism using MS. Finally, mice were euthanized and analysed for histology and enzyme assay. Created with Biorender.com*

To overcome such issues, chimeric mouse models, where the native mouse hepatocytes are replaced by human cells have been developed by our group and others (77). Such 'liver-humanized mice' may provide a faithful model to investigate metabolic liver diseases in actual disease-affected human hepatocytes. In paper 4, we described the successful generation of a novel mouse model for one of the urea cycle diseases; carbamoyl phosphate synthetase 1-deficiency, CPS1-D which is an autosomal recessive disorder with an occurrence estimated to be around 1 in 1,300,000 live births in the USA (88).

In this study, we used our FRGN mouse model, a mouse with a knockout of the *Fah*, *Rag2*, and *IL2rg* genes which was cross bread with Non-Obese Diabetic (NOD) mice (77). These FRGN mice readily accept human cells and can be highly repopulated with normal human hepatocytes or CPS1 deficient cells. Mice repopulated with normal hepatocytes has been previously described and served as controls for the present study.

Since the mouse is a model of a defect in *Fah*, the human disease corresponding to tyrosinemia type 1, as with the human patients the FRGN mouse needs to be maintained on the drug (2-(2-nitro-4-trifluoro-methylbenzoyl)1,3-cyclohexedione, NTBC), to limit lethal liver damage. A NTBC on/off cycle is used to generate loss of mouse hepatocytes which can be compensated by the transplantation and proliferation of FAH-proficient donor human hepatocytes.

Mice were transplanted with 1 million normal human hepatocytes (CPS1 proficient) or CPS1-D hepatocytes isolated from a patient who had undergone liver transplantation to correct the disease. Post transplantation of human cells, the level of humanization, or repopulation of the mouse liver with human cells was estimated by measuring the concenntration of human albumin in the blood of transplanted mice using ELISA. End stage analysis was performed on mice that have achieved a minimum of 4 mg/mL human albumin, which is estimated to correlate with ~80% repopulation with human hepatocytes (77). An ammonia challenge was performed on repopulated mice by infusing $[^{15}N]H_4Cl$ (4mmol/kg of body weight) through tail vein and blood ammonia levels were analyzed before infusion of ammonium chloride and 30 min post infusion. Basal ammonia levels in CPS1-D mice were higher than in control mice. Moreover, after the ammonium chloride challenge, CPS1-D mice displayed a significant delayed clearance of ammonia when compared to control animals. These observations from the mice are consistent with what is observed with human patients with the same disease.

Amino acids were also analyzed on mice repopulated with normal and CPS1 deficient cells. As also observed in human patients with this disease, the level of glutamine and glutamate were elevated in CPS1-D mice compared to control

mice that received CPS1-proficient human hepatocytes. Other amino acids such as citrulline, arginine, and alanine did not show a significant difference between the two groups.

The enzyme activity was measured to verify the CPS1 defect in the mice. The CPS1-D mouse showed only 20% of the enzyme activity when compared to mouse transplanted with healthy human hepatocytes. To determine if other urea cycle enzymes were affected in the CPS1-D mice, OTC (ornithine carbamoylase) gene expression was analyzed and found to be expressed at normal levels. Histological analysis shows CPS1 and OTC staining on highly humanized mice. The majority of the liver sections stained for human-specific CPS1 and OTC and remaining unstained mouse hepatocyte are shown in Figure 15.

In summary, CPS1-D human hepatocytes can be used to generate highly repopulated liver- humanized mice. The CPS1-D mouse exhibits characteristic symptoms of human CPS1-D. Liver-humanized mouse will be more informative than traditional mouse models and could play an important role in cellular and gene therapies (including the latest gene-editing technologies such as CRISPR). Since the target cell in liver-humanized mice is actual disease affected human hepatocytes, preclinical studies conducted on liver-humanized mice will be critical to investigate the safety and efficacy of gene editing therapies.

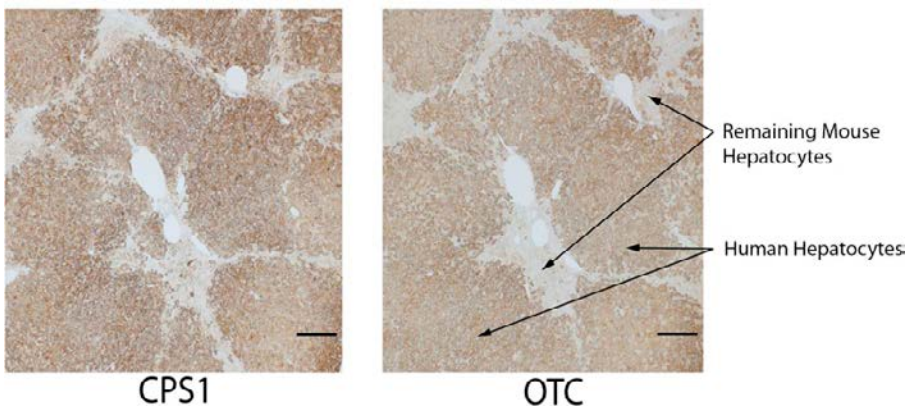


***Figure 15.*** *Histology. The liver sample collected from highly humanized mouse stained for CPS1 or OTC. The mouse hepatocytes and human hepatocytes can be clearly distinguished, showing human hepatocyte repopulation in mouse liver.*

## 16.5 Paper 5

**Evaluation of different routes of administration and bio-distribution of human amnion epithelial cells in mice**

The route of administration plays an important role in delivering a cell to the target organ, in our case, the liver. In this preclinical study, we intended to validate an efficient route of hAEC administration to the liver with minimal engraftment in other organs. The FRGN mouse model was used as a recipient for hAEC infusion and the bio-distribution was monitored. We characterized donor cells from three different hAEC cases by flow cytometry as previously described (with anti- CD326, CD49f, CD105, CD44, CD45, CD31).

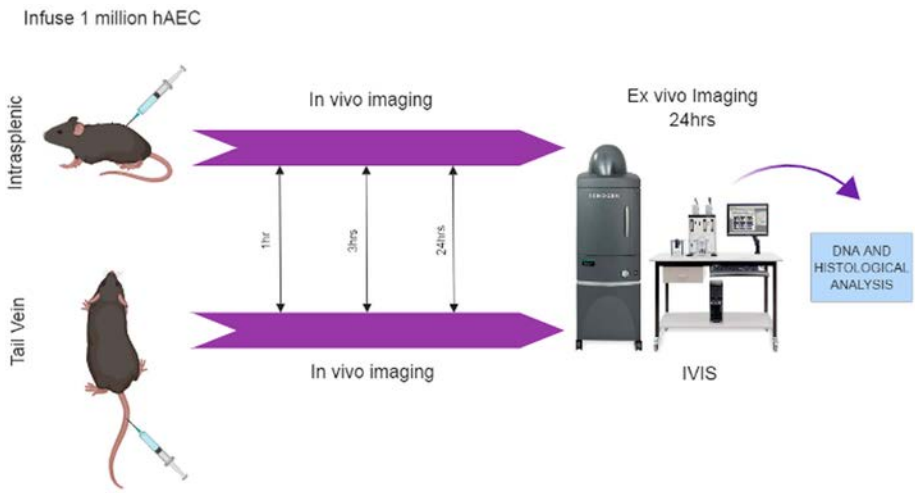


***Figure 16.*** *The project workflow. The FRGN mice were transplanted with hAEC (DiR labelled) via intra-splenic or tail vein. The distribution of hAEC were monitered on live mice using IVIS at 1, 3, and 24 h. The mice were euthanized at 24 h and organs were analysed for DNA and histology. Created with Biorender.com.*

To optimize cell delivery to the liver, we compared transplantation of hAEC via the spleen or systemically through the tail vein in mice. To trace the bio-distribution after transplantation, hAEC was labeled with a near infrared DiR dye and viewed with an *in vivo* imaging system (IVIS) at 1, 3, and 24 h. post-transplant. The localization of cells was observed in real-time, and in live animals. Twenty-four hours post-injection, the organs were collected and DNA was isolated. The presence of human hAEC DNA was quantified by PCR using a human-specific AluYb8 primer.

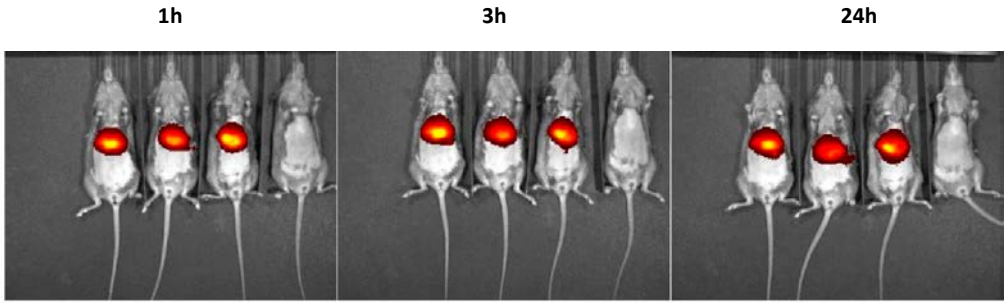


***Figure 17.*** *IVIS imaging. Transplanted hAEC via the spleen have localized to the liver as shown here at 1, 3, and 24 h. post-transplantation. RC.Srinivasan et al., Cytotherapy 2019. 21: 113-124*

The results clearly indicated the majority of the transplanted hAEC were detected in the liver when the transplants were conducted via the spleen, with little or no human DNA detected in other organs (heart, lungs, kidneys, and intestine) (Figure 17). On average $4.7 \times 10^5$ and $6.5 \times 10^4$, cells were tracked in the liver when transplanted via intrasplenic and tail-vein, respectively.

Histological studies confirmed hAEC integration into the mouse liver parenchyma, when injected through the spleen.

To conclude, our data support an efficient and safe delivery of hAEC into the liver achieved by intra-splenic infusion, a method frequently used in mice to model portal vein transplants in human patients. On the contrary, tail-vein infusion results in a different distribution pattern with cells mainly located in the lungs with significant localization in multiple other organs.

# 17 REFLECTION ABOUT ETHICAL CONSIDERATIONS WITHIN THE PROJECT

My project deals with hAEC as an alternative source for hepatocytes for treating liver metabolic diseases. The hAEC are isolated from full-term placenta after live birth. The placenta is normally discarded after the delivery, which makes them a minimal ethical concern. The human placentae utilized in my studies have been collected from healthy mothers after a caesarian section procedure and proper donation consent was signed. In paper 1, the ethical permit no 2015-419-31/4 was obtained.

The FRGN mouse model was used for the paper 4 and 5. The FRGN mouse has a mutation in *Fah* gene, which will cause liver injury. Lethal liver failure can be prevented by supplementing animals with NTBC drug. The mice receive humane care and provided with water and food accessible throughout the experiment. Mice approximately 6 weeks of age were transplanted with cells.

The aim of paper 3, is to analyze the bio-distribution of the hAEC after transplanting them through the spleen or intravenously. We have separate ethical permission to transplant hAEC through tail vein (B102-15) and spleen (70-15). The mice were humanely euthanized at the end of the experiment and organs were collected for further analysis. Animal experiments were required to investigate and optimize the route of administration of hAEC, to efficiently target the liver and to have a better understanding of the migration and engraftment of the cells. As a pre-clinical study, this data can be useful to the regulatory authorities to determine the safety of the procedure needed to take the hAEC to the clinic to treat patients with metabolic liver disease.

Finally, the PKU project (paper 3) required xenotransplants and was performed at the University of Pittsburgh. The ethical permit was approved by the same University (0905698B-12). The PKU mouse model was transplanted with hAEC directly into the liver multiple times during the first 28 days. Since human patients with inborn errors of metabolism may require to be treated at a very early stage, $Pah^{enu2}$ mouse was treated at an early stage (before weaning) to model the timing of a clinical transplant. Project results support the safety and efficacy of hAEC repeated transplants, supporting perinatal stem cell translation into the clinic.

# 18 FUTURE PERSPECTIVE

The research on hAEC over the last decade has led to a better understanding of their immunomodulation, immunosuppressive and *in vivo* differentiation ability. Our results have shown the potential of hAEC for liver cell therapy.

In paper 1, we have shown the isolation of hAEC with GMP grade reagents and we would like to move the isolation procedure to a GMP facility for banking of cells for future cell therapy. Our preclinical studies show that the cells we will isolate can be preserved and bio-banked for future cell therapy. Currently, we have an ethical permit to transplant hAEC into 10 patients to treat liver diseases that might otherwise be treated by hepatocyte transplantation.

Paper 2, the cryopreservation procedure for hAEC can be improved to increase the viability after thawing procedure. There are different cryopreservation media now available which can be used to reduce the cell loss during cryopreservation procedure. In the future, we would also like to culture the hAEC *in vitro* to proliferate and provide more cells for transplantation, but so far we have not succeeded. New technologies such as organoid culture might be helpful for cell proliferation without altering the characteristics of the hAEC.

In Project 3, we created liver-humanized mouse models of the human disease CPS1-deficiency. In future studies this highly human relevant model can be used to investigate the safety and efficacy of cellular tharapies such as hAE transplants, or gene or CRISPR-based therapies. We are also working on another liver humanized mouse model for Ornithine transcarbamylase, another urea cycle disorder. Treating and rescuing the mouse from metabolic liver diseases will be important preclinical studies and help us in translating hAEC to the clinic. As these mouse models are humanized with human hepatocytes the symptoms observed in the mice are consistent with what we see in patients.

# 19 ACKNOWLEDGEMENTS

I know acknowledgements is the only page that everyone reads, so I will try to cover everyone who helped me during my thesis.

**Dr. Prof. Stephen C. Strom**: It has been a great journey since I came to your lab. I have admired the way you respect and treat people. Thanks a lot for trusting me and motivating in all situations. I should be lucky to start my research career at your lab.

**Roberto Gramignoli and Kristina Kannisto**: My great co-supervisors. Every single technique that I do now was taught by you both. Thanks for the patience and teaching, and sharing all your knowledge.

**Mihaela Zabulica:** The CRISPR girl. We have traveled the whole Ph.D. studies together. Thanks for babysitting me with teaching the CRISPR technique. We share a lot of memories and of course a lot of chit-chatting and gossips. I eagerly waiting to see the small version of you. **Christina Hammarstedt,** our transplant expert. No animal project is possible without your presence. Thanks for singing for the mice and keeping them alive. I really appreciate you came all the way to India for me and hope you had a great time there. **Tingting Wu**, thanks a lot for helping me out with the lab and animal work.

**Ewa Ellis** and her group members **Carl Jorns, Helen Zemack, Heléne Johansson, Lisa Marie,** and **Ahmad Karadagi** ( thanks for putting up with me for W.B and your genuine smile every time we meet).

My pathology friends **Agatha, Magali, Anja, Rim, Christina Patlaka, Martin, Ghazal, Lisa, Marita, Laia, Massoud, Ali, Suchita, Joman, Ashish, Ahn Tran, Tuomas, Zurab, Lena Hernberg, Pernilla, Gareth, Maria Norgård, Maria Hortlund, Usha, Selvi, Chris, Francesco Ravaioli (bevilo tuto), Emanuel Kolanko, Chris, Lianne (Thanks for teaching us Organoids), Laia, Mikael Hammarstedt, Barbro, Annica, Raoul, Tarja, Arja Kramsu**.

**Åsa-Lena** for helping me out with all FACS studies and all stories that you tell me during the reading. **Mia Bjerke**, you have answered all my questions with a smiling face.

I would like to thank, the head of the Pathology division – **Göran Andersson** for being friendly and creating a wonderful work environment.

**Sougat Misra**: My mentor. Thanks for all the valuable comments and being a critic with me and helped me in almost everything from statistics to presentation.

**Arun Kumar**: It would have been hard without you here. I admired your dedication and focus on your research. Hope you will reach higher in your carrier. **Hanna Kann,** you are an awesome and genuine person. We will remember 'the Wednesday party' forever.

**Patricia**, my dear talkative friend, keep that smile on your face always and best of luck with your PhD defence.

**Flemingsberg gumbals**: You guys are more than friends. The Las Palmas trip was the best adventure trip we did together and infinite dinner and weekends together. Thanks, **Ram, Kala, Sharan, Sujatha, Roshan** and **Anjali**-the newcomer.

My Solna friends, **Sampath, Subbu, Swetha, Shuba, Balaji, Supria, Nari, Preeti, Kalai, Ekambar**, **Rameez**.

**Sarah and Hans:** You are our moral support in Sweden and thanks for guiding us and advising in many situations. I admire the way you handle life in a tough situation and being a good example for us.

Finally, Thanks to my parents; **Srinivasan** and **Nalini.** You have done everything that you can do for me. **Madhurya**, I'm lucky to have such a lovely wife. I did not know you two years ago, but now I cannot imagine anything without you. I have admired your patience and calmness (except the days you fight with me). My brother **Vasantharaman,** thanks for your support and I owe a lot to you.

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Arkitektkopia AB, Bromma, 2019